

**The Extent of Genotype-specific Humoral Immunity
Produced on Infection with Hepatitis C Virus:
Relevance for Serological Screening and Diagnosis.**

Judith Anne Neville

PhD
The University of Edinburgh
2003



Declaration

The research described in this thesis is solely the work of Judith Neville unless clearly indicated that another member of the Murex Diagnostics research team performed it. The author has composed the contents of this thesis.

Signature:

Date: 04 JUN 04

Acknowledgements

I would like to thank my supervisors Professor Peter Simmonds, Dr Sarah Howie and Professor Dorothy Crawford for their help and advice during this piece of work.

I would also like to acknowledge Dr Ian Pike, Dr Brian Rodgers and the Murex Biotech Ltd. research team for advice and supply of samples, test kits and reagents.

I am grateful to Dr A. El-Zayadi, Dr G.M. Dusheiko, Dr A.A. Saeed, Dr G.H. Haydon and Dr A. Widdell for sending samples to the Molecular Biology laboratory for serological analysis.

Proof reading was performed by Maureen O'Hara.

Many thanks go to the staff both past and present of the Molecular Virology Group and the Regional Virus laboratory for their help and support.

Finally thank you to my parents and friends for their patience, encouragement and love.

Murex Biotech Ltd and the Scottish National Blood Transfusion Service funded this work.

**“Perseverance is favourable”
Principle of I Ching**

TABLE OF CONTENTS

1	HEPATITIS C VIRUS.....	19
1.1	VIRION	19
1.2	SIMILARITY OF HCV TO OTHER VIRUS FAMILIES.....	19
1.3	VIRAL ORGANISATION, STRUCTURE AND FUNCTION	20
1.4	HCV RECEPTOR	23
1.5	HCV IN THE CELL	24
1.5.1	VIRAL KINETICS	25
1.6	GENOME VARIABILITY	25
1.6.1	CLASSIFICATION SYSTEM FOR HCV	29
1.7	PREVALENCE	32
1.7.1	INFECTION	36
1.7.2	SYMPTOMS AND MARKERS OF INFECTION	37
1.7.3	ASSOCIATED DISEASE	40
1.8	IMMUNE RESPONSE	41
1.9	TREATMENT	43
1.9.1	NEW THERAPIES	44
1.10	DETECTION.....	45
1.10.1	INITIAL SEROLOGY	45
1.10.2	CONFIRMATORY SEROLOGY.....	46
1.10.3	DETERMINATION OF CLEARANCE/ CHRONIC CARRIAGE.....	50
1.10.4	GENOTYPING	50
1.10.5	VIRAL LOAD TESTING	51
1.10.6	HCV CORE ANTIGEN.....	51
1.11	AIMS OF THE PROJECT.....	51
1.11.1	INVESTIGATION OF HCV GENOTYPE-SPECIFIC ANTIBODIES	51
1.11.2	INVESTIGATION OF LEVEL OF DETECTION OF HETEROLOGOUS HCV GENOTYPE INFECTION.....	52
1.11.3	TO INVESTIGATE IF THE USE OF HETEROLOGOUS ANTIGENS CAN DETECT INFECTIONS UNDETECTABLE BY CURRENT SEROLOGICAL METHODS, BUT DETECTED BY MOLECULAR BIOLOGY	52
1.11.4	TO INVESTIGATE IF USE OF HETEROLOGOUS HCV GENOTYPE ANTIGENS CAN RESOLVE CONFIRMATIONAL ASSAY INDETERMINATE SAMPLES..	52
1.11.5	INVESTIGATION TO DETERMINE IF HETEROLOGOUS HCV ANTIGEN ASSAYS CAN INCREASE SENSITIVITY OF DETECTION IN THE “WINDOW PHASE”	53

2	MATERIALS AND METHODS.....	55
2.1	POLYMERASE CHAIN REACTION	55
2.1.1	DETECTION OF VIRAL RNA BY USING PCR AMPLIFICATION	56
2.1.2	RESTRICTION FRAGMENT LENGTH POLYMORPHISM.....	61
2.2	CLONING.....	65
2.2.1	P-TAG VECTOR - LIGATOR KIT USE OF A-T PAIRING FOR INSERTION INTO VECTOR	65
2.2.2	PCR PREPARATION	66
2.2.3	LIGATION REACTION	66
2.2.4	TRANSFORMATION REACTION	66
2.2.5	MINI-PREP FOR EXTRACTION OF DNA.....	67
2.3	SEQUENCING	68
2.3.1	DNA PREPARATION FOR SEQUENCING REACTIONS.....	69
2.3.2	ANNEALING REACTION	69
2.3.3	DENATURING POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)...	70
2.3.4	USE OF DTP SEQUENCING FOR CORE SEQUENCES.....	70
2.4	USE OF RESTRICTION ENZYME SITES IN PRIMERS TO POSITION DNA SEQUENCE IN PLASMID.....	71
2.5	EXPRESSION OF RECOMBINANT PROTEINS.....	71
2.6	PREPARATION OF ANTIGEN COATED ELISA PLATES.....	72
2.6.1	ELISA FOR DETECTION OF ANTIBODY TO SINGLE ANTIGENS.....	72
2.6.2	ELISA FOR DETECTION OF ANTIBODY DIRECTED AGAINST GENOTYPE 3A CORE, NS3, NS4 AND NS5 ANTIGENS.....	73
2.6.3	QUANTIFICATION OF ANTIBODY LEVELS	74
2.6.4	PLATE TO PLATE VARIATION	78
2.7	COMMERCIAL ELISAS.....	80
2.7.1	MUREX VK48.....	80
2.7.2	ABBOTT 3 RD GENERATION ENZYME IMMUNOASSAY (EIA).....	81
2.7.3	ORTHO 3 RD GENERATION EIA	82
2.7.4	CHIRON RECOMBINANT IMMUNOBLOT ASSAY 3.0 (RIBA)	84
2.7.5	MUREX SEROTYPING ASSAY	85
2.8	STATISTICS	86
3	RESULTS 1.....	88
3.1	DETERMINATION OF THE GENOTYPE-COMMON AND GENOTYPE- SPECIFIC COMPONENTS OF ANTIBODY RESPONSE TO SINGLE ANTIGENS	88
3.2	METHODS	89
3.2.1	SAMPLES.....	89
3.2.2	ANTIGENIC SEQUENCE	89
3.2.3	ASSAY TECHNIQUES	90
3.2.4	QUANTIFICATION OF ANTIBODY LEVEL.....	90
3.2.5	CALCULATION OF THE GENOTYPE-SPECIFIC AND GENOTYPE-COMMON COMPONENTS OF THE ANTIGENIC RESPONSE.....	90

3.3	RESULTS	92
3.3.1	SEQUENCE ANALYSIS AND PHYLOGENETIC COMPARISON.....	92
3.3.2	GENOTYPE DEPENDENCE OF SEROLOGICAL REACTIVITY.	100
3.3.3	QUANTIFICATION OF ANTIBODY LEVELS.....	100
3.3.4	REPRODUCIBILITY OF RESULTS.....	106
3.3.5	CORRELATION BETWEEN REACTIVITY TO DIFFERENT ANTIGENS	106
3.3.6	RATIO OF REACTIVITY TO A HOMOLOGOUS GENOTYPE ANTIGEN: REACTIVITY TO A HETEROLOGOUS ANTIGEN.	110
3.4	DISCUSSION ON SINGLE ANTIGENS	113
3.4.1	ANTIGENIC VARIABILITY OF HCV	113
3.4.2	IMPLICATIONS FOR SCREENING ASSAYS	114
4	<u>RESULTS 2.....</u>	<u>117</u>
4.1	DETERMINING THE GENOTYPE-COMMON AND GENOTYPE-SPECIFIC COMPONENTS OF ANTIBODY RESPONSE TO COMBINED ANTIGENS.	117
4.2	METHODS	117
4.2.1	SAMPLES.....	117
4.2.2	ANTIGENIC SEQUENCE	118
4.2.3	ASSAY.....	120
4.2.4	MEASUREMENT OF ANTIBODY LEVEL	120
4.2.5	RATIO OF REACTIVITY	121
4.3	RESULTS	121
4.3.1	SEQUENCE	121
4.3.2	GENOTYPE DEPENDENCE OF SEROLOGICAL REACTIVITY AND QUANTITATION OF ANTIBODY LEVELS	123
4.3.3	CORRELATION BETWEEN REACTIVITY TO DIFFERENT ANTIGEN.....	123
4.3.4	RATIO OF REACTIVITY TO A HOMOLOGOUS GENOTYPE ANTIGEN: REACTIVITY TO A HETEROLOGOUS ANTIGEN.	127
4.4	DISCUSSION ON COMBINED ANTIGENS.....	127
5	<u>RESULTS 3.....</u>	<u>130</u>
5.1	INTRODUCTION	130
5.2	THEORETICAL UNDER-DETECTION OF ANTI-HCV ANTIBODY-POSITIVE SAMPLES OF OTHER GENOTYPES.	132
5.2.1	INTRODUCTION.....	132
5.2.2	SAMPLES.....	134
5.2.3	ASSAYS.....	134
5.2.4	METHODS	135
5.2.5	RESULTS	135
5.2.6	INTERPRETATION.....	138

5.3	SCREENING DONOR POPULATIONS IN CAIRO, EGYPT AND RIYADH, SAUDI ARABIA.....	139
5.3.1	INTRODUCTION.....	139
5.3.2	SAMPLES.....	140
5.3.3	ASSAYS.....	141
5.3.4	METHODS.....	141
5.3.5	RESULTS.....	142
5.3.6	DETERMINATION OF CUT-OFF VALUE FOR GENOTYPE 4A ANTIGEN ASSAYS.....	143
5.3.7	ORTHO 3 EIA.....	147
5.3.8	MUREX VK48.....	147
5.3.9	SEROLOGY RESULTS SINGLE ANTIGENS - BLOOD DONORS OF EGYPTIAN ORIGIN.....	147
5.3.10	SEROLOGY RESULTS SINGLE ANTIGENS - BLOOD DONORS OF SAUDI ARABIAN, YEMENIS, AND SUDANESE ORIGINS.....	149
5.3.11	REPRODUCIBILITY OF THE RESULTS.....	151
5.3.12	REACTIVITY TO GENOTYPE 1B ANTIGENS.....	154
5.3.13	CONDITION OF SAMPLES DURING TRANSIT.....	154
5.3.14	INTERPRETATION.....	155
5.4	SCREENING DONOR POPULATIONS IN PAKISTAN.....	156
5.4.1	INTRODUCTION.....	156
5.4.2	SAMPLES.....	156
5.4.3	ASSAYS.....	157
5.4.4	METHODS.....	157
5.4.5	RESULTS.....	158
5.4.6	INTERPRETATION.....	160
5.5	ANALYSIS OF RIBA CONFIRMATION TEST RESULTS IN SCOTTISH BLOOD DONORS.....	161
5.5.1	INTRODUCTION.....	161
5.5.2	SAMPLES.....	163
5.5.3	ASSAYS.....	164
5.5.4	METHODS.....	164
5.5.5	RESULTS.....	165
5.5.6	INTERPRETATION.....	167
5.6	SEROLOGICAL DIAGNOSTIC TEST RESULTS IN HEPATITIS PATIENTS FROM PAKISTAN.....	168
5.6.1	INTRODUCTION.....	168
5.6.2	SAMPLES.....	169
5.6.3	ASSAYS.....	169
5.6.4	METHODS.....	170
5.6.5	RESULTS.....	171
5.6.6	INTERPRETATION.....	178
5.7	SEROCONVERSION PANELS.....	179
5.7.1	INTRODUCTION.....	179
5.7.2	SAMPLES.....	180
5.7.3	ASSAYS.....	181
5.7.4	METHODS.....	181
5.7.5	RESULTS.....	182
5.7.6	INTERPRETATION.....	191

5.8	SAMPLES PCR POSITIVE BUT CONVENTIONAL ANTIBODY ASSAY NEGATIVE OR DISCREPANT.....	193
5.8.1	INTRODUCTION	193
5.8.2	SAMPLES.....	193
5.8.3	ASSAYS.....	194
5.8.4	RESULTS	194
5.8.5	INTERPRETATION.....	194
6	<u>DISCUSSION OF FINDINGS FROM EXPERIMENTAL WORK... 196</u>	
6.1	INVESTIGATION OF THE LEVEL OF DETECTION OF HETEROLOGOUS HCV GENOTYPE INFECTION	198
6.2	TO INVESTIGATE IF THE USE OF HETEROLOGOUS ANTIGENS CAN DETECT INFECTIONS UNDETECTABLE BY CURRENT SEROLOGICAL METHODS, BUT DETECTED BY MOLECULAR BIOLOGY.....	198
6.3	TO INVESTIGATE IF USE OF HETEROLOGOUS HCV GENOTYPE ANTIGENS CAN RESOLVE CONFIRMATIONAL ASSAY INDETERMINATE SAMPLES	199
6.4	INVESTIGATION IF HETEROLOGOUS HCV ANTIGEN ASSAYS CAN INCREASE SENSITIVITY OF DETECTION IN THE “WINDOW PHASE”....	199
6.5	THE OVERALL AIM IS TO ASCERTAIN WHICH COMBINATION OF ANTIGENS BEST DETECTS INFECTION WITH ALL GENOTYPES.....	199
6.6	NUCLEOTIDE AMPLIFICATION TECHNOLOGY	200
6.7	HOW HAS THIS WORK ADVANCED THE CURRENT UNDERSTANDING OF HCV SEROLOGY?.....	200
	<u>REFERENCES</u>	201
	<u>APPENDIX 1.....</u>	217
	<u>APPENDIX 2.....</u>	219
	<u>APPENDIX 3.....</u>	223

Tables

TABLE 2.1 COORDINATES OF PRIMERS AND SIZE OF AMPLICON PRODUCED AND CONDITIONS REQUIRED	60
TABLE 3.1 DIVERGENCE BETWEEN GENOTYPE IN THE RECOMBINANT ANTIGENS	93
TABLE 3.2 FREQUENCY OF REACTIVITY TO CORE, NS3 AND NS5 PROTEINS. .	101
TABLE 3.3 CORRELATION BETWEEN REACTIVITY TO DIFFERENT ANTIGENS...	109
TABLE 3.4 TYPE-SPECIFIC AND TYPE-COMMON SEROLOGICAL REACTIVITY TO HCV CORE, NS 3 AND NS5 ANTIGENS	112
TABLE 4.1 DIVERGENCE BETWEEN GENOTYPE IN THE RECOMBINANT ANTIGENS	122
TABLE 5.1 DESCRIPTION OF THE SOURCE AND NUMBER OF SERA SAMPLES AVAILABLE FOR STUDY WITH THE SINGLE ANTIGEN AND COMBINED GENOTYPE 3A SCREENING ELISA.....	131
TABLE 5.2 SUMMARY DATA FOR INITIAL TESTING OF THE GENOTYPE 4A CORE ANTIGEN WITH COMMERCIAL HCV EIA SERONEGATIVE BLOOD DONORS.	144
TABLE 5.3 SUMMARY DATA FOR INITIAL TESTING OF THE GENOTYPE 4A NS3 ANTIGEN WITH COMMERCIAL HCV EIA SERONEGATIVE BLOOD DONORS.	145
TABLE 5.4 SUMMARY DATA FOR INITIAL TESTING OF THE GENOTYPE 4A NS4 ANTIGEN WITH COMMERCIAL HCV EIA SERONEGATIVE BLOOD DONORS.	146
TABLE 5.5 THE SEROLOGICAL REACTIVITY FOUND IN THE EGYPTIAN BLOOD DONORS TESTED ON TYPE 4A ANTIGEN.	148
TABLE 5.6 SAMPLES WHICH WERE COMMERCIAL ASSAY UNREACTIVE, BUT WHICH HAVE GENOTYPE-SPECIFIC REACTIVITY WITH ONE OR MORE SINGLE ANTIGENS.....	150
TABLE 5.7 FREQUENCY OF REACTIVITY OF SAMPLES WITH SINGLE ANTIGEN TESTS IN THE FIELD IN SAUDI ARABIA AND IN THE LABORATORY AT DARTFORD.	152
TABLE 5.8 REACTIVITY TO GENOTYPE 1B COMMERCIAL ASSAY AND TO THE GENOTYPE 3A EQUIVALENT OF THE COMMERCIAL ASSAY IN PAKISTANI BLOOD DONOR SERUM SAMPLES.	159
TABLE 5.9 THE REACTIVITY OF RIBA INDETERMINATE SAMPLES FOR GENOTYPE 1B, 2B AND 3A NS3 ANTIGEN.....	166
TABLE 5.10 REACTIVITY TO NS3 ANTIGEN OF GENOTYPE 3A AMONG LIVER UNIT PATIENTS, WHICH WERE SERUM RNA NEGATIVE OR SERUM RNA POSITIVE. REACTIVITY DEFINED AS AN O.D OF GREATER THAN 0.6	172
TABLE 5.11 HCV PCR STATUS AND MUREX VK48 COMMERCIAL ASSAY RESULTS AMONG LIVER UNIT PATIENTS AND BLOOD DONOR CONTROLS FROM KARACHI.....	174
TABLE 5.12 DISCREPANT SAMPLES FROM LIVER UNIT PATIENTS FOUND TO BE ANTI-HBV AND ANTI-HCV ANTIBODY NEGATIVE BY TESTING IN PAKISTAN.....	176
TABLE 5.13 DISCREPANT SAMPLES FROM LIVER UNIT PATIENTS FOUND TO BE ANTI-HBV ANTIBODY POSITIVE AND ANTI-HCV ANTIBODY NEGATIVE BY TESTING IN PAKISTAN.	177
TABLE 5.14 THE SEROLOGICAL RESULTS FOR THE SEROCONVERSION PANEL OF SAMPLES FROM INDIVIDUALS A, B AND C.	183

TABLE 5.15 THE SEROLOGICAL RESULTS FOR THE SEROCONVERSION PANEL OF SAMPLES FROM INDIVIDUALS D, E AND F.....	185
TABLE 5.16 THE SEROLOGICAL RESULTS FOR THE SEROCONVERSION PANEL OF SAMPLES FROM INDIVIDUALS G, H AND I.	187
TABLE 5.17 THE SEROLOGICAL RESULTS FOR THE SEROCONVERSION PANEL OF SAMPLES FROM INDIVIDUAL J.	189
TABLE 5.18 THE SEROLOGICAL RESULTS FOR THE CONTROL SAMPLES PROVIDED BY DR WIDDELL.....	190

Figures

FIGURE 1.1 HCV GENETIC ORGANISATION AND POLYPROTEIN PROCESSING ..	21
FIGURE 1.2 HCV REPLICATION IN THE CELL	26
FIGURE 1.3 PHYLOGENETIC ANALYSIS OF AMPLIFIED HCV NUCLEIC ACID SEQUENCES	30
FIGURE 1.4 GLOBAL PREVALENCE OF HCV PUBLISHED BY THE WORLD HEALTH ORGANISATION WEEKLY EPIDEMIOLOGICAL RECORD. N° 49, 10 DECEMBER 1999.	34
FIGURE 1.5 TREE DIAGRAM SHOWING THE DISTRIBUTION OF HCV GENOTYPES AROUND THE WORLD.	35
FIGURE 1.6 MARKERS OF INFECTION, PRESENCE OF ANTI-HCV ANTIBODY, HCV RNA AND ALT LEVEL OVER TIME SINCE INFECTION	39
FIGURE 1.7 THE COMPONENTS OF A SELECTION OF THE MOST COMMON COMMERCIALY AVAILABLE DIAGNOSTIC EIA ASSAYS FOR DETECTION OF ANTI-HCV ANTIBODY.	47
FIGURE 1.8 ANTIGENIC REGIONS USED IN COMMERCIAL SCREENING ASSAYS ..	48
FIGURE 1.9 CONSTRUCTION OF THE CONFIRMATORY RIBA 2.0 AND 3.0 ASSAYS	49
FIGURE 2.1 PRIMERS USED FOR AMPLIFICATION OF NCR, CORE, AND NS3 AND NS5 REGIONS.....	59
FIGURE 2.2 ANALYSING THE COMBINATION OF THE HaeIII/RsaI AND MvaI/HinfI	63
FIGURE 2.3 RFLP FRAGMENTS AFTER DIGESTIONS WITH BstUI AND SrfI ENZYMES, ALLOWING DISTINCTION BETWEEN A AND B SUBTYPES.	64
FIGURE 2.4 CORRELATION OF ANTIBODY IN A SAMPLE AGAINST OPTICAL DENSITY MEASURED.	76
FIGURE 2.5 CONCENTRATION OF ANTIBODY TO OPTICAL DENSITY MEASURED IN THE STANDARD SAMPLE.....	77
FIGURE 2.6 CONSTRUCTION OF AN ENZYME LINKED IMMUNOSORBENT ASSAY	79
FIGURE 3.1 THE INFERRED AMINO ACID SEQUENCE OF THE RECOMBINANT CORE PROTEIN ANTIGENS (AMINO ACIDS 1-140).	94
FIGURE 3.2 THE PHYLOGENIC RELATIONSHIP PREDICTED USING UPGMA ANALYSIS OF THE CORE REGION	95
FIGURE 3.3 THE INFERRED AMINO ACID SEQUENCE OF THE RECOMBINANT NS3 PROTEINS (AMINO ACIDS 1360-1454).	96
FIGURE 3.4 THE PHYLOGENETIC RELATIONSHIP PREDICTED USING UPGMA ANALYSIS OF THE NS 3 REGION BETWEEN THE SAMPLES AMPLIFIED AND THE RECOMBINANT CLONES.....	97
FIGURE 3.5 THE INFERRED AMINO ACID SEQUENCE OF THE RECOMBINANT NS5 REGION PROTEIN ANTIGENS (AMINO ACIDS 2238-2321).....	98
FIGURE 3.6 THE PHYLOGENETIC RELATIONSHIP PREDICTED USING UPGMA ANALYSIS OF THE NS 5 REGION	99
FIGURE 3.7 DISTRIBUTION OF ANTIBODY REACTIVITY OF SERA FROM INDIVIDUALS INFECTED WITH 1B, 3A AND 4A VIRUS WITH GENOTYPE 1B AND 4A CORE ANTIGEN	103
FIGURE 3.8 DISTRIBUTION OF ANTIBODY REACTIVITY OF SERA FROM INDIVIDUALS INFECTED WITH 1B, 3A AND 4A VIRUS WITH GENOTYPE 1B, 3A AND 4A NS3 ANTIGEN.....	104

FIGURE 3.9 DISTRIBUTION OF ANTIBODY REACTIVITY OF SERA FROM INDIVIDUALS INFECTED WITH 1B, 3A AND 4A VIRUS WITH GENOTYPE 1B AND 3A NS5 ANTIGEN.....	105
FIGURE 3.10 MEASUREMENT OF THE REPRODUCIBILITY OF MEASUREMENT OF LEVELS OF ANTIBODY TO TYPE 1B CORE PROTEIN.....	107
FIGURE 3.11 MEASUREMENT OF THE REPRODUCIBILITY OF MEASUREMENT OF LEVELS OF ANTIBODY TO TYPE 4A CORE PROTEIN.....	108
FIGURE 3.12 RATIOS OF REACTIVITY OF TYPE 1B AND 4A SERA TO TYPE 1B /TYPE 4A CORE ANTIGENS.	111
FIGURE 4.1 THE AMINO ACID SEQUENCE OF THE NS4 BRANCHED PEPTIDES (REGION 1 AMINO ACIDS 16910 1708 AND REGION 2 1710-1729).	119
FIGURE 4.2 DISTRIBUTION OF ANTIBODY REACTIVITY FROM SAMPLES OF GENOTYPE 1 (N=36), GENOTYPE 2 (N=7), GENOTYPE 3 (N=23) AND GENOTYPE 4 (N=10) TO ANTIGENS OF THE GENOTYPE 1 VK48 SCREENING ASSAY.	124
FIGURE 4.3 DISTRIBUTION OF ANTIBODY REACTIVITY FROM SAMPLES OF GENOTYPE 1 (N=36), GENOTYPE 2 (N=7), GENOTYPE 3 (N=23) AND GENOTYPE 4 (N=10) TO ANTIGENS OF THE GENOTYPE 3 BHC129 RECOMBINANT ASSAY.....	125
FIGURE 4.4 THE CORRELATION BETWEEN ANTIBODY REACTIVITY TO GENOTYPE 3A ANTIGENS AND REACTIVITY TO GENOTYPE 1B ANTIGENS.	126
FIGURE 4.5 RATIO OF REACTIVITY TO TYPE 1B ANTIGENS COMPARED TO TYPE 3A ANTIGENS.....	128
FIGURE 5.1 ANTIBODY LEVELS IN 111 HCV GENOTYPE 1B INFECTED ANTI- RHESUS D RECIPIENTS.	136
FIGURE 5.2 ANTIBODY LEVELS IN 111 HCV GENOTYPE 1B INFECTED ANTI- RHESUS D RECIPIENTS DIVIDED BY FIVE TO REPRESENT A FIVE-FOLD REDUCTION IN ASSAY SENSITIVITY.....	137
FIGURE 5.3 CORRELATION OF RESULTS OF GENOTYPE 4A CORE REACTIVITY MEASURED IN SAUDI ARABIA COMPARED TO REPEATED REACTIVITY RESULTS MEASURED IN DARTFORD	153

Table of Abbreviations

AMV RT	Avian Myeloblast Virus Reverse Transcriptase
ALT	Alanine Aminotransferase
BSA	Bovine Serum Albumin
BVDV	Bovine Viral Diarrhoeal Virus
cDNA	Complementary Deoxyribonucleic acid
DDT	Dithiothreitol
DEPC	Diethyl pyrocarbonate
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
(dATP, dTTP, dCTP dGTP)	deoxyadenosine, deoxyguanosine, deoxythymidine, deoxycytidine collectively dNTP
ddNTP	dideoxyadenosine, dideoxyguanosine, dideoxythymidine, dideoxycytidine
<i>E.coli</i>	Escherichia coli
EDTA	Ethylene diaminetetra-acetic acid
ELISA	Enzyme Linked Immunosorbent Assay
EIA	Enzyme Immuno Assay
HBV	Hepatitis B Virus
HCV	Hepatitis C Virus
HGV	Hepatitis G Virus
HIV	Human Immunodeficiency Virus
HVR	Hypervariable region
IPTG	Isopropyl-thiogalactoside
IgG	Immunoglobulin G
LB broth/agar	Luria- Bertani Broth/Agar see appendix 1
LDL	Low density lipoprotein
MHC	specific major histocompatibility complex
MoI	Measure of Infectivity
NCR	Non Coding Region of the HCV Genome
NS3	Non Structural Region 3 of the HCV Genome
NS4	Non Structural Region 4 of the HCV Genome
NS5	Non Structural Region 5 of the HCV Genome

OD	Optical Density
OPD	1,2 -phenylenediamine dihydrochloride
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerise Chain Reaction
PKR	ds RNA dependent protein kinase
Poly A	Poly adenylic acid
RNA	Ribonucleic acid
RFLP	Restriction Enzyme Fragment Length Polymorphism
SDS	Sodium Dodecyl Sulphate
SVR	sustained virological response
TE	Tris (hydroxymethyl)methylamine hydrochloride/ Ethylen diaminetetra-acetic acid
TMB	3,3'5,5'- tetramethylbenzidine
TNF α	Tumour Necrosis Factor α
Tris-HCl	Tris(hydroxymethyl)methylamine hydrochloride
UTR	Untranslated regions
X-gal	5-bromo-4-chloro-3-indolyl-b-d-galactopyranoside.

Abstract

Blood transfusion services around the world screen donations for antibodies to hepatitis C virus (HCV). One potential problem of this is that the antibody response only becomes detectable several weeks after infection and potentially infectious donations in the "window period" go undetected. The screening ELISA tests are based upon genotype 1 sequence. Six distinct HCV genotypes exist, which show approximately 30 % amino acid sequence divergence in their encoded proteins. Sequence variability is not uniform across the genome, being greatest in the regions encoding envelope proteins and most restricted in the core gene. Sequence variation in the antigenic determinants in core, NS3, NS4A/B and NS5A/B proteins, may produce genotype-specific antibodies which do not cross-react with the antigens used in current screening assays for detection of antibody to HCV. Reduction in test sensitivity caused by genotype-specific reactivity may particularly affect detection of antibody around the time of seroconversion, as antibody reactivity is likely to be weak and restricted to a limited number of epitopes.

The extent of type-specific and type-common components of the humoral immune response were investigated by measuring the antibody levels in samples of each genotype with type-homologous and type-heterologous antigens. Reactivity directed against the individual genotype 1 antigens used in a commercially available anti-HCV screening assay (VK48; Murex Biotech) was compared with corresponding core, NS3 and NS4A/B antigens of genotype 3, and NS3, NS4A/B and NS5A/B antigens from genotype 4 using a panel of samples from individuals infected with different genotypes (genotype 1: n=33, 3: n=34 and 4: n=43). A combined ELISA was subsequently assembled using the core, NS3 and NS5A/B recombinant proteins and NS4A/B synthetic peptide of genotype 3a sequence, and serological reactivity in this ELISA compared quantitatively with reactivity in the (type 1-based) Murex VK48 assay, using a similar panel of samples from individuals infected with different HCV genotypes. The overall proportion of type-specific reactivity in the combined ELISAs was 46% (with the type-common component making up the other 54%). Type-specificity of reactivity to each region depended on the extent of amino acid divergence between genotypes, with the more conserved core region eliciting 26% type-specific reactivity, compared with 60% and 62-77% to the NS3 and NS5A/B regions.

To investigate whether antigenic variability influenced the effectiveness of type-1 based serological assays for screening, blood donor samples collected in regions where non-genotype 1 HCV predominated, such as genotype 3 infection in Pakistan and genotype 4a infection in Egypt and the Middle East, were screened by ELISAs based on genotype 3a or 4a antigens. Additionally, samples from seroconversion panels, individuals identified with a serum positive PCR result, but a commercial assay negative result and individuals with cryptogenic hepatitis, were tested for reactivity to antigens of genotype 2, 3 or 4. Although several samples were identified with type-specific reactivity confined to non-type 1 antigens, none were PCR positive. These results therefore indicate that while some (past, resolved) HCV infections may not be detectable using commercially available type 1-based screening assays, the lack of detectable viraemia in the samples provides no evidence at present that these serological "misses" risk the safety of blood transfusion. However, the results do indicate that a proportion of past HCV infections may remain undetected by current diagnostic methods.

Chapter 1

Between October 1995 and June 1998, this work was carried out as part of a research group sponsored by Murex Biotech Ltd. Apart from clearly stated exceptions, such as cloning and expression of the recombinant antigens used, the work described in this thesis was carried out by the author. Dr L Prescott, Dr C Blake and Mr N Adams carried out the isolation and amplification of the HCV sequences. The expression of recombinant proteins and coating of ELISA plates with antigen was carried out by Mr S Tucker, Mr P Egan and Ms R Mason under the supervision of Dr I Pike and Dr B Rodgers.

1 Hepatitis C Virus

The Hepatitis C Virus (HCV) was discovered by the research group headed by Michael Houghton at the Chiron Corporation in conjunction with the Centres for Disease Control in 1989 (Choo et al., 1989). It is a small enveloped, ribonucleic acid (RNA) virus that shows some similarities to plant pestiviruses and animal flaviviruses, but is more closely related to another newly discovered virus, hepatitis G virus (HGV) (Linnen et al., 1996) also described as GB virus C (Simons et al., 1995). Transmission is by parenteral routes and the virus is prevalent throughout the world. Infection results in a wide spectrum of disease from mild jaundice to eventual hepatocellular carcinoma. Approximately 55-85% of those infected become chronic carriers and current treatment with interferons, anti-virals and combination therapies have a limited effect. HCV infection is a major economic problem in terms of lost working hours, cost of treatment and death.

1.1 Virion

Electron microscopy revealed that the virus exists as two types of particles, small particles of 30-35 nm or 45-55 nm and large particles of 50-75 nm (Kaito et al., 1994; Takahashi et al., 1992); (Li et al., 1995) (Prince et al., 1996; Shimizu et al., 1996). The small particles are assumed to be non-enveloped nucleocapsids and the larger particles complete virions.

The measurement of buoyant density in sucrose gives a range of values from 1.06 g/cm to 1.17 g/cm (Bradley et al., 1991; Carrick et al., 1992; Kanto et al., 1994; Kanto et al., 1995), again suggesting several sizes of particles. The low-density particles are more infectious than the high-density particles and tend to be associated with β lipoprotein (Thomssen et al., 1992; Thomssen et al., 1993). Addition of anti- β lipoprotein to serum of an infected individual precipitates HCV (Thomssen et al., 1992). The higher density particles may represent naked capsids and enveloped virions bound to immunoglobulins (Choo et al., 1995; Hijikata et al., 1993b; Kanto et al., 1995).

1.2 Similarity of HCV to other Virus families

HCV has been classed as a separate genus Hepacivirus in the Flaviviridae family because it has a similar hydrophobicity profile but a limited sequence identity with the two other genera of the family, the flaviviruses and the pestiviruses. The newly discovered GBV-A, GBV-B viruses of primates and the human HGV/ GBV-C

virus are also classified as Flaviviridae because of their similarity with HCV. The flaviviruses contain viruses transmitted by ticks and mites and other insect vectors, including yellow fever virus, dengue virus and Japanese Encephalitis virus. The pestiviruses include viruses of farmed animals, bovine viral diarrhoea virus, classical swine fever and border disease virus of sheep. HCV shares a similar highly conserved 5' untranslated region with pestiviruses but not with flaviruses. Similarities with other viruses exist in the serine protease, helicase and polymerase genes.

1.3 Viral organisation, structure and function

The positive stranded RNA virus genome consists of approximately 9400 nucleotides that form an open reading frame that codes for a 3300 amino acid polyprotein. Host signal peptidases and two virally encoded protease enzymes process this protein. The genome is organised into a N-terminal or 5' non coding or untranslated region (5'NCR or 5'UTR), followed by a nucleocapsid or core protein, two envelope genes designated E1 and E2, 6 non-structural genes NS2, NS3, NS4A, NS4B, NS5A, NS5B and a 3' or C-terminal untranslated region (Figure 1.1).

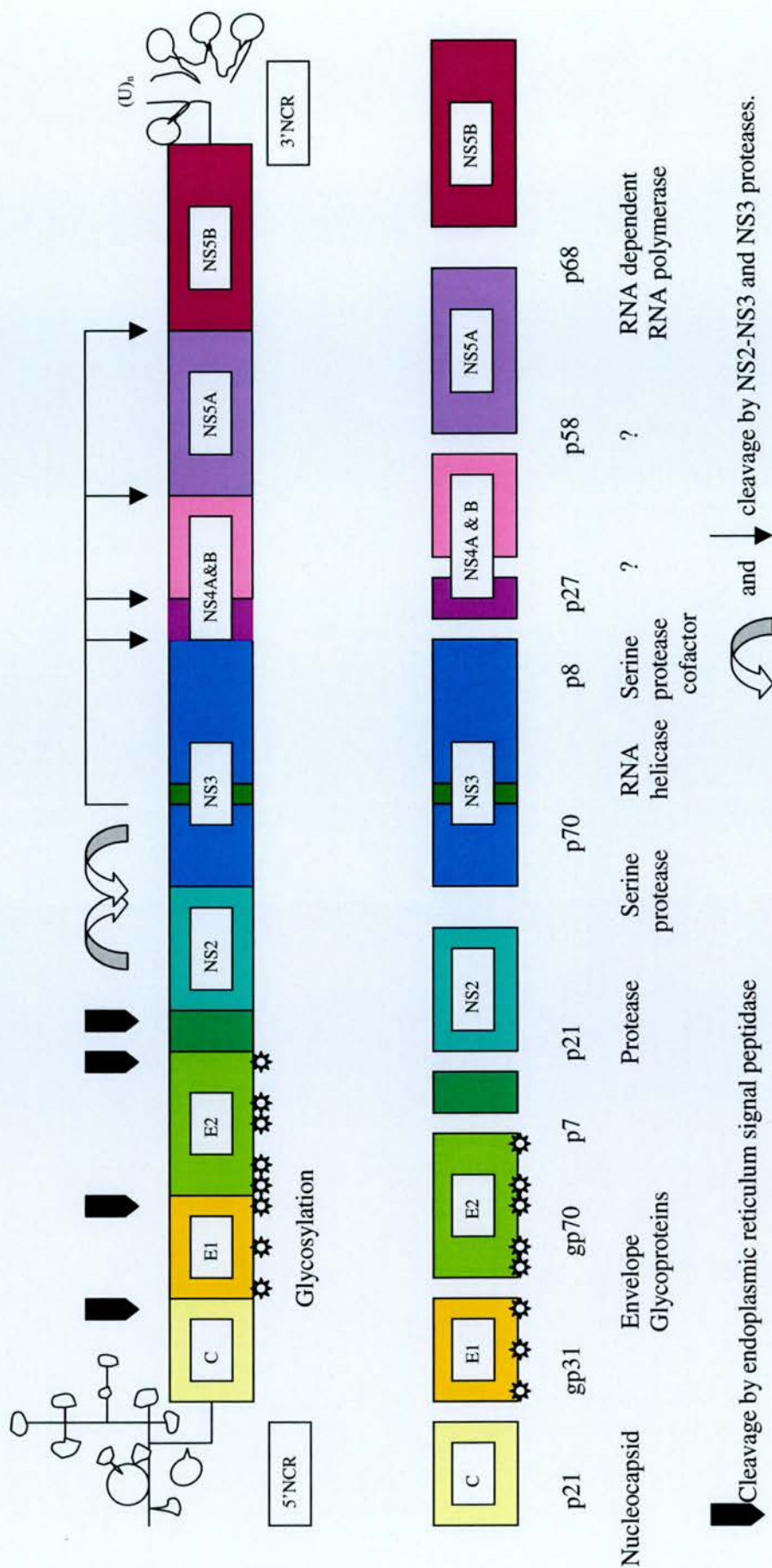


Figure 1.1 HCV Genetic Organisation and Polyprotein Processing

The NCR is an internal ribosome entry site (Tsukiyama Kohara et al., 1992) (Wang et al., 1993) essential for cap-independent translation of RNA. The 5' non-coding region is 341 nucleotides in length it is highly conserved between all the HCV genotypes and subtypes. The region forms a complex RNA secondary structure including a large conserved stem loop (125-323 nucleotides), a pseudo-knot (305-311 and 325-331 nucleotides), and a small stem-loop containing an AUG codon (331-354 nucleotides).

The first cleavage product of the polyprotein is the highly basic core protein, forming the major constituent of the nucleocapsid (Yasui et al., 1998). Envelope proteins (E1 and E2) are highly glycosylated type 1 transmembrane proteins. Protein p7, located at the carboxyl terminus of E2, is a highly hydrophobic polypeptide which functions as an ion channel (Pavlovic et al., 2003). Most of the non-structural (NS) proteins 2–5B (the term indicates that these proteins are not expected to be constituents of the virus particle) are required for replication of the viral RNA (Lohmann et al., 1999). NS2 and the amino-terminal domain of NS3 constitute the NS2–3 proteinase, catalysing cleavage at the NS2/3 site (Grakoui et al., 1993a; Hijikata et al., 1993a; Hirowatari et al., 1993). NS3 is both a serine-type proteinase responsible for cleavage at the NS3/4A, NS4A/B, NS4B/5A and NS5A/B sites and, a NTPase/helicase, essential for translation and replication of the HCV genome (Eckart et al., 1993; Grakoui et al., 1993b; Gwack et al., 1996). In addition, NS3 may have other properties involved in interference with host cell functions like inhibition of protein kinase A-mediated signal transduction or cell transformation (Borowski et al., 1996). NS4A is an essential cofactor of the NS3 proteinase and is required for efficient polyprotein processing (Bartenschlager et al., 1994; Failla et al., 1994). The function of the hydrophobic NS4B is so far unknown. NS5A is a highly phosphorylated protein. The phosphorylation is mediated by an as yet unknown cellular kinase (Ide et al., 1997). The role NS5A may play in RNA replication is so far not known, but based on analogy with other RNA viruses, where phosphoproteins are important regulators of replication; one could assume that NS5A plays a similar role. NS5A appears to be involved in resistance of the infected cell to the antiviral effect of IFN. At least for some HCV isolates NS5A is able to bind to dsRNA dependent protein kinase (PKR), blocking the translational reduction in the IFN-treated cell (Gale et al., 1997).

NS5B was identified as the RNA-dependent RNA polymerase (RdRp) (Al et al., 1998; Lohmann et al., 1997) (Behrens et al., 1996).

The 3' untranslated region (UTR) can be divided into 3 domains by virtue of the sequence composition. The first domain constitutes the most heterogeneous sequence and is followed by a domain composed of a poly-A or poly-UC run of variable length. The third domain consists of a highly conserved 3' tail of 98 nucleotides, which is important for initiation of viral RNA replication (Tanaka et al., 1996) (Kolykhalov et al., 1996).

1.4 HCV Receptor

Several cell surface molecules have been proposed as the HCV receptor: the low density lipoprotein (LDL) receptor (Agnello et al., 1999), (Monazahian et al., 1999), a tetraspanin molecule designated CD81 (Pileri et al., 1998) and human scavenger receptor class B type 1 (SB-BI) (Scarselli et al., 2002; Bartosch et al., 2003). Due to the lack of cell culture systems for HCV, alternative approaches were used to establish the link between HCV and the proposed receptors. Agnello and colleagues demonstrated that HCV and other Flaviviridae including GB virus C /HGV and bovine viral diarrhoeal virus (BVDV) enter cells in culture mediated by the LDL receptor. Endocytosis occurred in cell culture line G4 and HepG2 cells when HCV was complexed with very low-density lipoprotein (VLDL) and LDL. Antibodies could inhibit the intake of HCV to the anti-LDL receptor, by cyclohexanedione that is known to disrupt the LDL receptor-binding site and by competitive inhibition with large concentrations of LDL and VLDL (Wunschmann et al., 2000).

Pileri's group demonstrated that CD81 binds to expressed recombinant E2 protein (Pileri et al., 1998). CD81 is a widely expressed 25 kD molecule and a member of the tetraspanin super family (Levy et al., 1998) expressed on various cell types including hepatocytes and B-lymphocytes. This cell surface protein spans the cell membrane four times forming two extra cellular loops. The major extra cellular loop is highly conserved in humans and chimpanzees the only two species HCV is known to infect. Binding of HCV to CD81 was mapped to the major extra cellular loop of CD81. The binding affinity of recombinant E2 to the recombinant large extra cellular loop of CD81 is 1.8 nM at 25 °C and 9.1 nM at 37 °C (Petracca et al., 2000). This is similar to the binding affinity 1-4 nM reported for Human Immunodeficiency Virus (HIV) gp 120 binding to CD4

(Moore et al., 1991). If the binding affinity values calculated in vitro are representative of the situation in vivo, neutralising antibodies to the envelope region would have to have affinity constants of higher than 10^{-9} M to prevent viral attachment.

CD81 has a relatively poor ability to internalise ligands and it may serve as an HCV attachment receptor rather than a receptor for virus entry.

The LDL receptor and CD81 attachment operate independently of each other with antibodies to one failing to block attachment to the other (Wunschmann et al., 2000).

Scarselli's group found that binding of recombinant E2 to CD81 was isolate specific however E2 recognition by human hepatoma cell lines was not. They isolated the receptor responsible for E2 binding to the cell line as SB-B1. They were able to demonstrate that the binding was selective since neither mouse SB-B1 nor the closely related human scavenger receptor DC36 were able to bind E2. Recognition of E2 by SR-B1 could be competed out by the addition of monoclonal antibodies specific for the E2 HVR (Scarselli et al 2002).

1.5 HCV in the cell

Hepatocytes represent the primary site of HCV replication. Although extracted peripheral blood mononuclear cells (PBMC) contain HCV RNA, (Bouffard et al., 1992; Muller et al., 1993; Schmidt et al., 1997) it is unclear if HCV replicates in PBMC in vivo (Afonso et al., 1999; Fong et al., 1991; Lanford et al., 1995; Wang et al., 1992). HCV RNA has also been found in T and B-lymphocytes (Blight et al., 1994; Bouffard et al., 1992; Zignego et al., 1992).

It is difficult to determine the sites of active replication as this depends on the identification of negative sense strands of RNA. The difficulty arises because all methods of detection require synthesis of a negative strand. Methods have been developed where specific primers or highly strand-specific rTth (recombinant *Thermus thermophilus* DNA polymerase can be used to detect the presence of negative strands (Yuan H et al., 2003). Our current understanding of the molecular mechanisms of HCV replication is based on comparison with the closely related flavi- and pesti-viruses and on the characterisation of recombinant HCV proteins. The presumed life cycle of HCV includes: binding to cell surface receptor and internalisation into the host cell; liberation of the genomic RNA from the virus particle into the cytoplasm; IRES mediated translation of the input RNA; processing of the polyprotein; utilization of the

input plus-strand for synthesis of a minus-strand RNA intermediate; production of new plus-strand RNA molecules which in turn can be used for synthesis of new minus strands for polyprotein expression or packaging into progeny virions and release of virus from the infected cell (Figure 1.2).

1.5.1 Viral Kinetics

HCV infection is a highly dynamic process with a viral half life of only a few hours and average daily virion production and clearance rates of up to more than 10^{12} (Neumann et al., 1998).

1.6 Genome Variability

RNA virus replication requires RNA polymerase, which is prone to a high error rate. This means that there are number of mismatches of base between the template RNA strand and the newly synthesised strand. Mutations can occur for many reasons including depurination, deamination, polymerase slippage and oxidative damage to bases. These errors remain uncorrected since no proof reading activity has been reported for RNA polymerases (Holland et al., 1982). There is a bias towards base transitions, i.e. pyrimidine to pyrimidine, purine to purine rather than to transversions - pyrimidine to purine, purine to pyrimidine. This may be caused by G to U mis-pairing (Both et al., 1983). The average error frequencies of virus RNA replicase and reverse transcriptase is of the order of 10^{-3} to 10^{-5} per site (Holland et al., 1992), yielding 10-100 viable replacements per site per year. Therefore, most RNA viruses exist as a collection of closely related sequences or quasispecies.

The reported nucleotide sequences of HCV show a varying degree of divergence from each other (Choo et al., 1991). Variation is seen between sequences isolated from different individuals and among different isolates from the same individual (Okamoto et al., 1991). The nucleotide sequence mutation rate was calculated for the complete genome of HCV as 1.44×10^{-3} base substitutions per site per year (Okamoto et al., 1992a). However, the variation was not uniformly distributed over the genome. The fidelity in several sites remained constant; others contained several substitutions while yet other regions appeared to be mutational hot spots.

Whether a given nucleotide substitution results in a viable mutant is site dependent; different regions of the HCV genome vary in their level of genetic conservation. Highly conserved areas include the 5' NCR and the core protein. Nucleotide substitutions in these regions are less likely to be viable, because changes may result in an altered structure that affects the function. This will apply to the RNA structure of regions with a replication function and to the regions where the function of the translated protein is disrupted. Other regions of the genome are more tolerant of nucleotide substitution.

There are two regions of the envelope genes that are highly variable in their nucleotide sequence. They have been called hypervariable region 1 (HVR1) and hypervariable region 2 (HVR 2). HVR1 is located at amino acid position 396-407 and HVR2 at amino acid position 380-436 in the E2 envelope glycoprotein (Weiner et al., 1992). These regions show similarity with the V3 loop of gp120 in HIV which also displays a high degree of variability, and it has been suggested that this variation is associated with escape from neutralising antibody binding (Kato et al., 1998; Weiner et al., 1992). The method of introduction of mutations in these regions is different from the rest of the genome. Throughout the genome the ratio of transitions to transversions is 3.92 but in the HVR it is 1.10. Also, analysis of variation in complete genomes found 62% of changes occurred in the 3rd nucleotide position of a codon, but in the HVR all the nucleotide changes were in the first or second codon positions. In an infected individual, the variation among quasispecies gives rise to populations of viruses that have different properties. There is a range of replicative efficiencies among the quasispecies and not all quasispecies are infective (Sugitani and Shikata, 1998). Studies have indicated that quasispecies differ with respect to the alanine amino-transferase (ALT) elevations they

induce, which is indicative of liver damage and some data suggest that greater diversity in HCV quasispecies correlates with more severe liver disease (Gonzalezperalta et al., 1996; Hayashi et al., 1997; Yuki et al., 1997). Quasispecies also appear to differ in their response to IFN α therapy (Mizokami et al., 1994; Pawlotsky et al., 1998) which reduces the viral sequence diversity as viral replication decreases (Gonzalezperalta et al., 1996). After IFN α therapy, different HCV quasispecies may become dominant. Patients with more than two major quasispecies tend to respond poorly to IFN α therapy (Gonzalezperalta et al., 1996), (Toyoda et al., 1997). Different quasispecies may be found in different locations within an individual. Sequence variation has been found between the liver and the plasma (Maggi et al., 1997) and it is possible that in this case mutations have given a selective advantage for individual tissues or cell types. The quasispecies profile in an individual also often changes after liver transplantation. This implies a role for the liver, as well as host immune responses, in selection of predominant quasispecies (Lawal et al., 1997; Yun et al., 1997). Approximately 90% of liver transplant recipients become reinfected, however, few develop severe HCV associated liver disease. In vertical transmission the predominant viral sequence in the child may not have been the predominant one in the mother at the time of the transmission (Kudo et al., 1997).

Again this shows that the possession of the diverse gene pool of quasispecies makes HCV very adaptable to environment changes.

Artefactual genome variation may be introduced to a specific sequence by laboratory methods used for sequencing the RNA. All methods of amplifying the RNA sequence utilise enzymes that have a degree of replication error. Substitutions can be introduced during copying of virus RNA by reverse transcriptase or during the 25-50 cycles of replication by Taq DNA polymerase, producing combined error rates in DNA clones of $2.5\text{-}5 \times 10^{-3}$ depending on sequence and reaction conditions. Therefore, the inferred RNA sequence derived may include several different bases to the original RNA template extracted. Laboratory methods to obtain a given sequence should involve repeating the sequencing reaction several times and combining the results to obtain a consensus sequence and careful interpretation of sequence data must be applied (Simmonds, 1993).

1.6.1 Classification system for HCV

Initially published sequences of complete HCV genomes were compared using computer programmes based on mathematical modelling that estimated the variation or pair wise distances between two sequences and predicted phylogenetic relationships. Using a model for molecular evolution (Felsenstein, 1988). HCV was shown to consist of isolates which shared approximately 88%-100% sequence identity, subtypes which shared 74-86% identity and genotypes which shared 54-72% identity (Figure 1.3) (Simmonds et al., 1993a; Simmonds et al., 1994). Six major genotypes of HCV exist with more than 80 subtypes. Additionally, analysis was carried out on the E1, core, and NS5 regions which showed that analysis of NS5 region nucleotide positions 7975 to 8196 completely concurred with sequence analysis of complete genomes. Thus complete genome sequencing was not necessary for genotyping analysis (Simmonds et al., 1994).

Sequence analysis found that several mutations were shared by isolates of the same genotype. This allowed methods of identification of the genotype or subtype to be devised which did not require full-length sequencing. Several methods were developed including, type-specific primers for amplification of the 5'NCR and core regions (Chayama et al., 1993; Okamoto et al., 1992b), restriction enzyme digestion of PCR products (Davidson et al., 1995; McOmish et al., 1993; McOmish et al., 1994; Murphy et al., 1994) and labelled hybridisation probes (Stuyver et al., 1993b; Van Doorn, 1994). Overall concordance between the different genotyping methods exceeds 93% (Lau et al., 1995). The serotype of infection, which normally concurs with the genotype, can be detected by determining type-specific antibody to core or NS4 regions. This will be discussed in section 1.10.4.

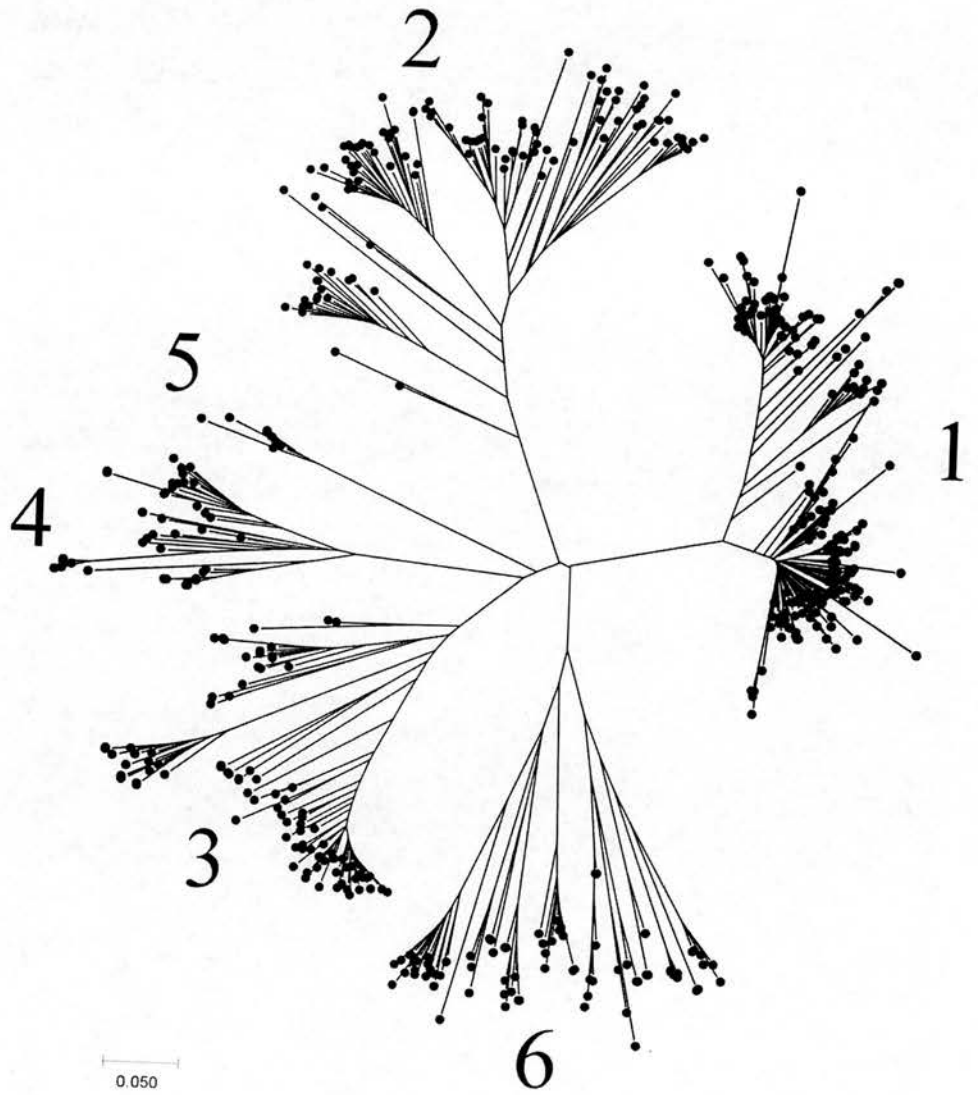


Figure 1.3 Phylogenetic analysis of amplified HCV nucleic acid sequences

In a clinical setting genotyping is carried out by amplification of the 5'NCR region by PCR after infection is confirmed. This is because methods which utilise the 5'NCR are more rapid and RNA detection is frequently higher for the 5'NCR than for other regions of the genome (Bukh et al., 1992;Castillo et al., 1992;Xu et al., 1994). Genotype-specific sequence polymorphisms exist throughout the 5'NCR except for a highly conserved 60-nucleotide region at the 3' terminus. The genotyping methods utilise the genotype-specific sequence polymorphisms to deduce the genotype of the virus. The line probe assay (LiPA) involves the hybridisation of 5'NCR RT-PCR products to type-specific oligonucleotides under conditions where single base mismatches prevent hybridisation (Stuyver et al., 1993a;Vandoorn et al., 1994), the Trugene HCV 5'NC Genotyping kit (Visible Genetics, Toronto, Canada) (Ansaldi et al., 2001, Ross et al, 2000) is based on direct sequencing of PCR amplicons and sequence comparison with a reference database, while for restriction fragment length polymorphism (RFLP) analysis, virus genotype is predicted from the electrophenotypes produced from RT-PCR products after restriction digestion (Davidson et al., 1995;McOmish et al., 1993;McOmish et al., 1994). The accuracy of the methods, RFLP and LiPA, is approximately 96% and 84% respectively for the prediction of virus type with higher accuracies for distinguishing subtypes 1a/1b, 2a/2b and 3a/3b (Smith et al., 1995).

Phylogenetic analysis found no evidence of recombination between HCV genomes (Simmonds et al., 1994). Recombination has been described in two Honduran patients (Yun et al., 1996) where the core sequence of genotype 1 was found with sequence analysis and type-specific core primers, and genotype 3 found by sequence analysis of E2 and NS5 regions. This may have been a mixed infection where the type-specific primers for core of type 3 did not bind to the genotype 3 sequence present, and genotype 1 sequence did not amplify in E2 and NS5 regions. If recombination does take place then it would be exceptionally rare, since for recombination to occur two viruses of separate genotypes must infect the same cell. The low incidence of HCV infection and the restricted genotype distribution in most communities make such an event unlikely, although an exception might be haemophiliacs who have been multiply infected with mixtures of virus genotypes over many years through regular infusions of blood products (Jarvis et al., 1994).

1.7 Prevalence

HCV infection has been reported from countries throughout the world. The World Health Organisation estimates 200 million people are carrying the virus. The prevalence in the UK and USA is between 0.1-1%. In Scotland alone 10 thousand people are on the national infection database and double that number are suspected to be infected (Scottish Centre for Infection and Environmental Health web site). Many parts of the developing world have a higher prevalence of disease. The World Health Organisation lists the following countries as having a prevalence of greater than 10%: Bolivia, Burundi, Cameroon, Egypt, Guinea, Mongolia, Rwanda and Tanzania. Furthermore, Gabon, Libya, Papua New Guinea, Surinam; Vietnam and Zaire have prevalences of between 5-10% (World Health Organisation Weekly Epidemiological Record. N° 49, 10 December 1999 [Figure 1.4]). In some countries, for example, Pakistan, prevalence as high as 40% has been reported in rural areas (Dr A Saeed personal communication). A proportion of these infections has to be attributed to mass vaccination campaigns where sterilization of equipment was not carried out between vaccinations.

The true prevalence of HCV infection in many countries may actually be higher than official figures since many of the prevalence studies were carried out in blood donor screening centres. Blood donors are screened by interview to reject those who have engaged in any practices which are associated with risk factors for infection with HIV or hepatitis viruses, such as injecting drug abuse, tattooing, body piercing, homosexual sexual practices and multiple sexual partners.

The genotype of HCV found in each region of the world differs with rarely more than 3 types or subtypes prevalent in any particular location. Genotype 1 is ubiquitous and accounts for two-thirds of HCV-infected individuals in the United States, with approximately equal numbers infected with genotypes 1a and 1b. 10% of the US infected population carry genotype 2, and 6% are infected with genotype 3. Approximately 10% are infected with mixed genotypes, and the genotype cannot be determined in the remainder (Lau et al., 1996). As in the USA, genotype 1 (specifically 1b) is predominant in Europe. Genotypes 2 and 3 are typically found in 15% to 30% of infected Europeans. Genotype 3 is very common in Scandinavia and Scotland, where 40% of HCV infections are genotype 3. Genotype 3 is also prevalent in the Far East

(especially in Thailand), Pakistan, parts of India and in Australia. Genotype 4 is found almost exclusively in the Middle East. Genotype 5 is the most prevalent infection in South Africa, and genotype 6 is mainly found in Hong Kong (Figure 1.5) (McOmish et al., 1994; Mellor et al., 1995). In Scotland 50% of infections are genotype 1 (90% 1a and 10% 1b), 10% are type 2 and 40% are type 3 (McOmish et al., 1993). In the United States infection is most common in the 30 to 50 year age group. Prevalence of infection is very high among intravenous drug abusers and haemophiliacs who received clotting factor preparations prior to the implementation of viral inactivation steps (Jarvis et al., 1994).

Global prevalence of Hepatitis C Based on published data, update 1999

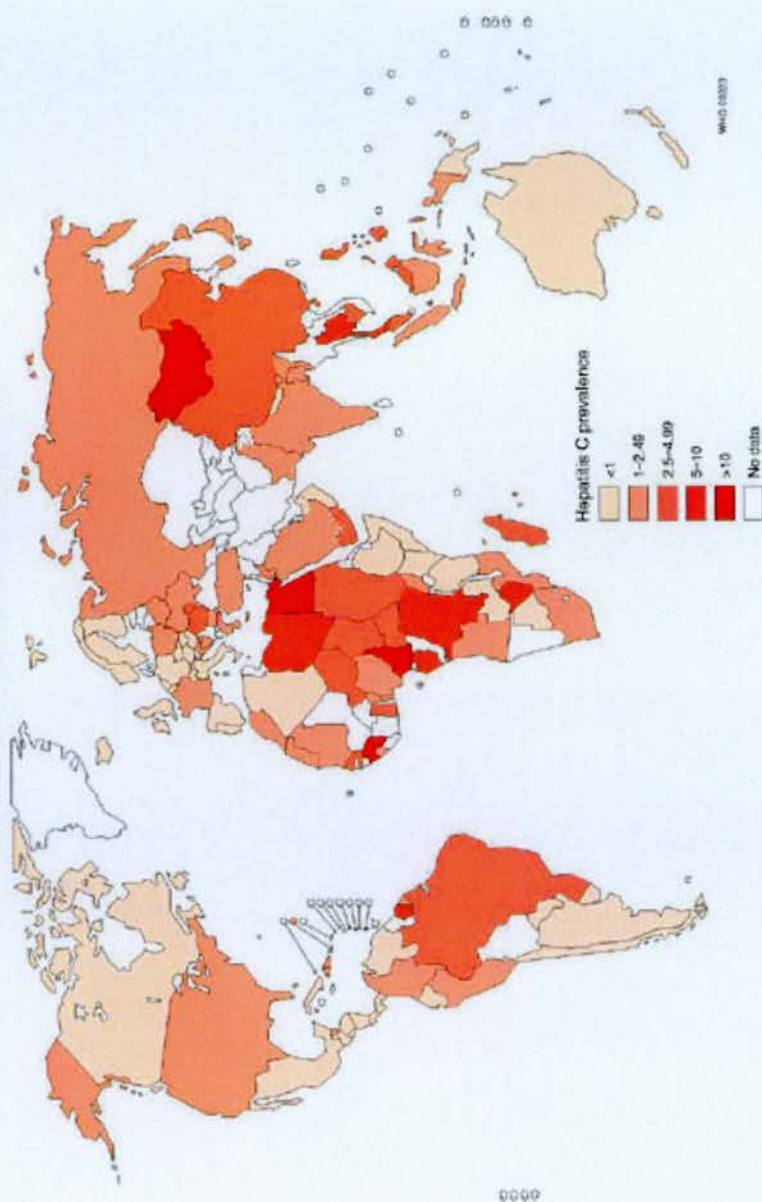


Figure 1.4 Global Prevalence of HCV published by the World Health Organisation Weekly Epidemiological Record. N° 49, 10 December 1999.

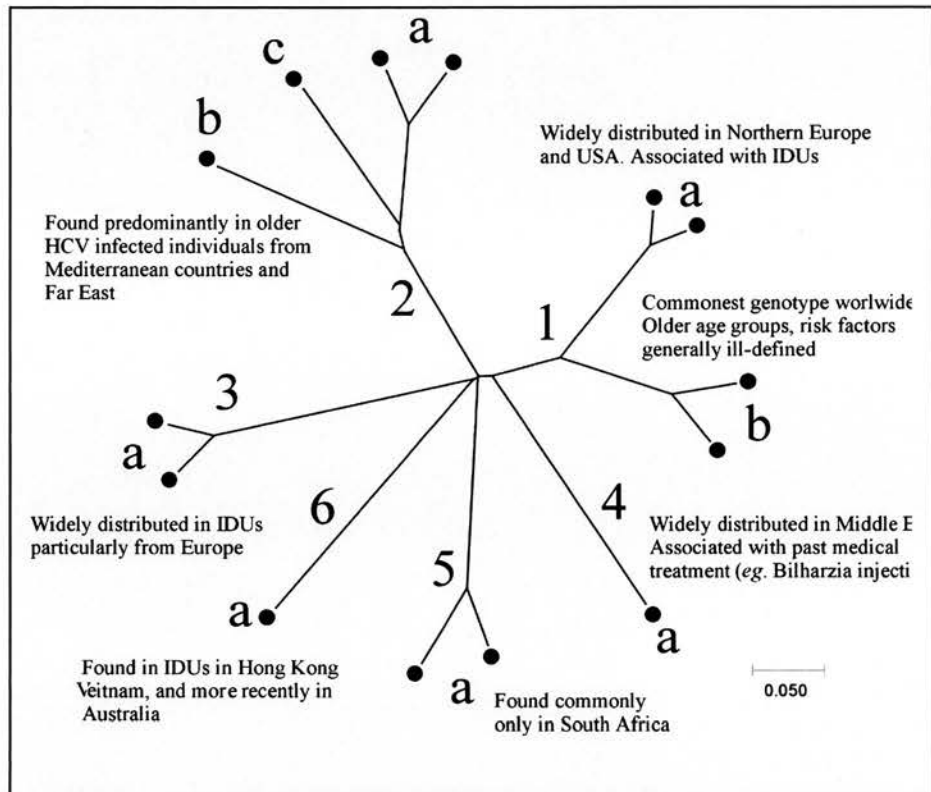


Figure 1.5 Tree Diagram showing the distribution of HCV genotypes around the world.

1.7.1 Infection

HCV infection is acquired by exposure to infected blood, or bodily fluids. Risk factors for HCV infection are transfusion of blood or blood products, transplantation of solid organs from an infected donor, injecting drug abuse, use of unsterile medical equipment, occupational exposure to blood, and birth to an infected mother or sex with an infected partner.

Following the introduction of HCV screening the incidence of infection following transfusion or blood product is very low. In the US prescreening the rate of infection was 1 per 200 units of blood now following screening and nucleic acid detection the rate is less than 1 per 1 000 000 units (Centers for Disease Control report).

Prior to virus inactivation steps (heat treatment, pasteurization and solvent – detergent treatment) by blood product manufacturers many people receiving blood products were exposed to HCV infection. Anti-RhD inoculations following birth of a Rhesus D positive child to a Rhesus D negative mother caused many women to become infected. Many haemophiliacs, thalassemics, and people with hypogammaglobulinaemia also became infected during the course of their treatment with blood products. Reinfection was observed in haemophiliacs, with different genotypes of the virus found in the same individual. A Dutch study (Vrieling et al., 1995) suggested that 81% of recipients of PCR positive blood components became HCV infected.

The major cause of new infections in the developed world is injecting drug abuse and use of unsterilised syringes is the major cause in the developing world.

The prevalence of HCV in different age groups shows three different trends associating with different geographic regions suggests different transmission profiles (Walsey et al., 2000). The first pattern is observed in the United States where infection in those younger than 20 is low, there is an increased amount in the middle age range and low rate in the elderly. This indicates a HCV infections occurred in the recent past, for example drug abuse in the 1960-1980s. There is a predominance of genotype 3 infection in these more recent infections compared to the genotype 1a in the older population. In France the trend is genotype 1a and 3a infection is associated with injecting drug abuse and genotype 1b nosocomial infection (Elghouzzi et al., 2000). Another profile which is observed in Italy and Japan where the prevalence is low in the young and younger adults but high in the elderly, this suggests most infections occurred 30-50 years ago (Guadagnino et al., 1997). This appears to correlate well with immunisation programmes around the

time of the second world war. The third profile which is evident in Egypt is prevalence steadily increasing with age indicating high rate of past and recent infection. This appears to correlate with a large reservoir of infected people, who were infected during the schistosomiasis programme, transmitting the infection by current unsterile medical practices.

The Infectivity in needle stick exposure is about 1.8% and there is no recommended prophylactic treatment (Memon et al, 2002, Wasley A et al., 2000). Treatment with anti-virals is not recommended and the American Advisory Committee on Immunisation Practices do not advise the use of anti-HCV serum globulin in infection prophylaxis following accidental exposure. Transmissions via blood splashes to the eye have been reported (Sartori et al, 1993), but these represent a few isolated occurrences.

Cases of sexual and vertical transmission have been reported, but they are thought to represent only a small fraction of infections (Capelli et al, 1997). There is no evidence that the virus can be spread by contact with the saliva or breast milk from an infected individual, and transmission by insects or other animal vectors is thought unlikely.

Infected individuals, once traced, are offered counselling and given advice on prevention of spread of the disease to household contacts and others. Infected people are requested to refrain from donations of blood, tissues, organs and semen. They are advised not to share toothbrushes and razors with others. There are no contrary guidelines with respect to pregnancy, breast-feeding and sexual contact within a current monogamous relationship, although the possibility of sexual and vertical transmission should be explained.

1.7.2 Symptoms and Markers of Infection

Hepatitis C has a mean incubation period of 7 weeks with a range of between 3 and 20 weeks. However, during this time markers of infection can be detected. HCV RNA can be detected within 3 weeks of infection (Farci et al., 1991; Thimme et al., 2001). Anti-HCV antibody can be detected, with 3rd generation assays, within approximately 12 weeks following infection. The liver enzymes alanine and aspartate transferase become elevated with peak increases 10 fold higher than baseline (Thimme et al., 2001; Alter et al, 2000) (Figure 1.6).

In about 20-33% of individuals, symptoms or jaundice appear during acute infection, these symptoms generally only last for between 2 and 12 weeks. Patients who develop chronic infection are less likely to have symptoms and jaundice than those with acute resolving infection (Alter et al, 2000).

In 5-45% of cases, acute, self-limiting infection results, the HCV RNA becomes undetectable and the ALT levels gradually return to normal (Takaki et al. 2000). At least 55% develop chronic HCV infection, where the symptoms of acute infection resolve, but the HCV RNA remains detectable, and in most cases, the ALT remains elevated. Although, the factors which determine chronic infection are not yet clearly defined, they may be due to the virus heterogeneity, viral proteins inhibiting immune response pathways, weak immune responses, activation of the CD4 T helper 2 pathway, rather than the T-helper 1 pathway, HLA haplotype, viral kinetics overwhelming the host responses, compartmentalisation in the liver and cytokine profile of T helper response. Viral genotype has been shown not to affect the progression of disease although the genotype may influence the outcome of interferon therapy (Bukh et al., 1992; Liang et al., 2000). Of chronically infected individuals in this "healthy carrier" state, approximately 33% have normal ALT levels while others have normal levels with intermittently raised episodes (Conry-Cantilena et al., 1996). Liver biopsies in patients with normal ALT levels show histological changes in virtually all patients, demonstrating that ALT level does not correlate well with histological finding. Long-term follow up shows that most patients with progressive liver disease who develop cirrhosis have prominent intermittent ALT elevations. Approximately 20% of chronic carriers develop symptoms and signs of liver disease. Patients with higher ALT are more likely to have symptoms. The frequent symptoms experienced include: fatigue, headache, nausea, poor appetite, myalgia, arthralgia, feverishness, weakness and weight loss. These symptoms are rarely incapacitating, but decrease quality of life. Chronic HCV infection can lead to cirrhosis and end-stage liver disease. This can develop rapidly or slowly within 2-3 decades.

The levels of viral RNA in the liver do not correlate with the histological findings of damage to the liver and, therefore, there must be several host factors that influence progression to cirrhosis. These factors are a combination of intrinsic and extrinsic factors. Of the intrinsic factors, the gender (Kenny-Walsh, 1999), immunocompetence and age at time of infection (Sasaki et al., 1997) appear to be the most important in determining the spread of progression to cirrhosis. The individual's HLA type, underlying disease (such as diabetes, haemophilia and haemochromatosis) are intrinsic factors that may also affect progression to cirrhosis. The dominant extrinsic factor is use of alcohol, but other, unproven, factors include smoking, environment and geographic location

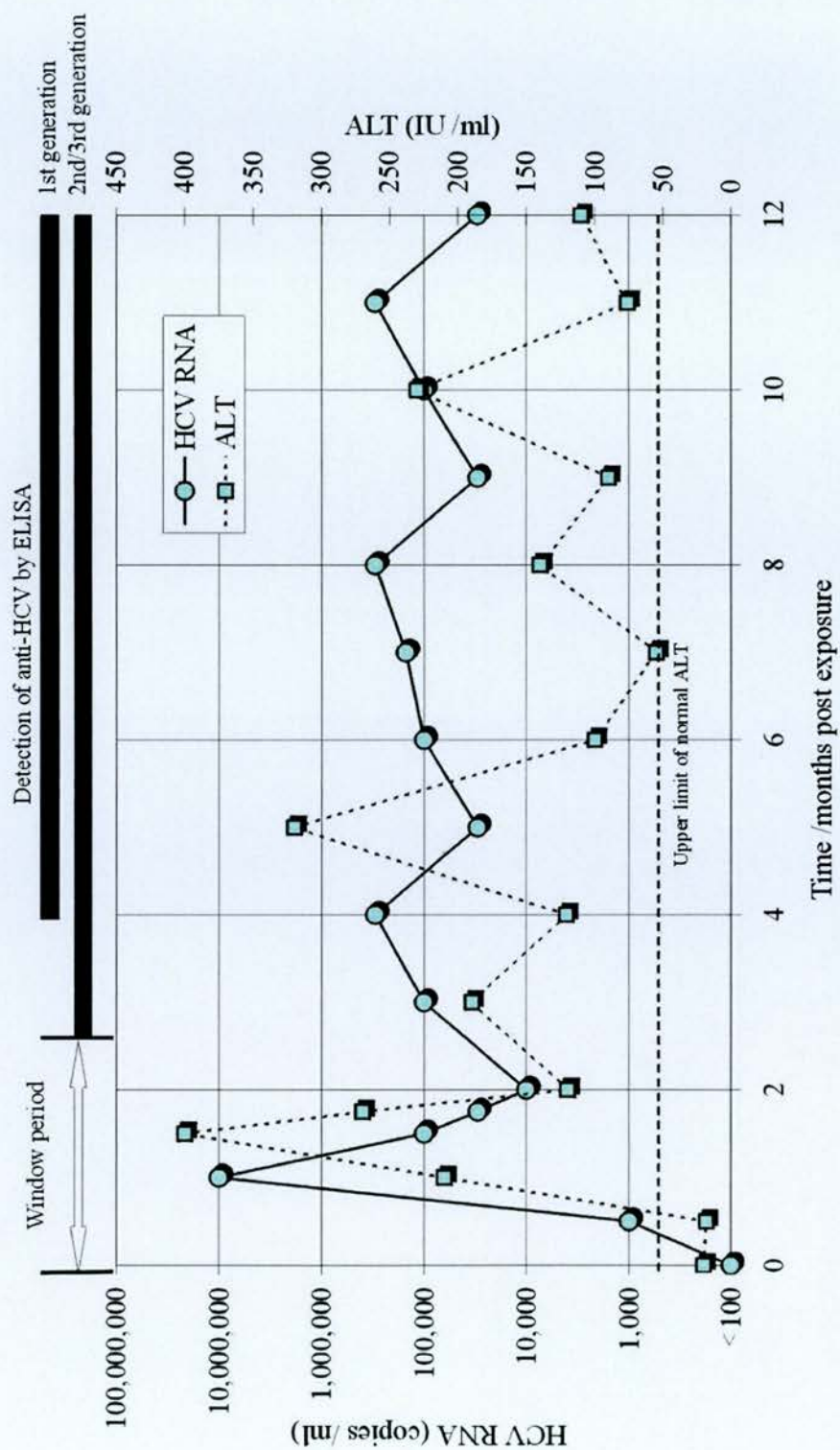


Figure 1.6 Markers of Infection, presence of anti-HCV antibody, HCV RNA and ALT level over time since infection

1.7.3 Associated Disease

Other extrahepatic conditions have been associated with infection including; Essential mixed cryoglobulinemia, Lymphoma, Glomerulonephritis, Porphyria cutanea tarda, Diabetes mellitus, Corneal ulceration, Uveitis, Autoimmune phenomena, Sialadenitis, Peripheral neuropathy (Gumber et al. 1994, Pawlotsky JM et al. 1995, Koff et al. 1995).

Hepatitis C virus-related cryoglobulinemia is the most common extrahepatic manifestation. Testing of patients with chronic hepatitis C shows that up to half have low levels of cryoglobulins. Blood tests show the presence of globulins that precipitate in the cold that include rheumatoid factor, complement, and complexes of HCV RNA and anti-HCV. (Ikeda et al., 1991) Symptoms of cryoglobulinemia including fatigue, skin rash, purpura, arthralgias, renal disease and neuropathy are only present in 1% of those infected. Skin biopsies show leukocytoclastic vasculitis. This disease can lead to progressive renal disease and severe vasculitic complications.

The disease responds to interferon therapy but relapse is common at the end of treatment (Misiani et al., 1994).

Hepatitis C has also been linked to B-Cell non Hodgkin lymphoma in several case series and cohort studies (Hausfater et al. 2001). The majority of patients with HCV related lymphoma have a history of cryoglobulinemia and it is unclear if this is a malignancy or a consequence of cryoglobulinemia and chronic B cell stimulation.

Glomerulonephritis can occur with immune complexes deposition in the kidneys, this condition also benefits from antiviral treatment of the underlying hepatitis C.

It is unclear if other conditions; sero-negative arthritis, keratoconjunctivitis sicca (Haddad et al., 1992), lichen planus (Tanei et al., 1995), neurological disorders (Heckmann et al. 1999) and porphyria cutanea tarda (Bonkovsky et al. 1998) are related to hepatitis infection or are consequences of underlying liver disease or immune stimulation from the chronic infection.

Porphyria cutanea tarda occurs in many liver diseases and is commonly found in hepatitis C infection (Bonkovsky et al. 1998). The disease responds to phlebotomy to deplete Iron levels.

1.8 Immune response

The immune response to HCV is polyclonal and multi specific, both in terms of antibody and cellular immune responses

HCV antibodies develop between 7 to 31 weeks after infection (Chien et al., 1992). The humoral immune response is multi-specific and targets the core, envelope NS3 and NS4 (Akatsuka et al., Simmonds et al., 1993). There is limited evidence from chimpanzee studies and tissue culture systems that neutralising antibody exists which is directed at the envelope proteins particularly the hypervariable region (Farci et al., 1996). Immunisation of chimpanzees with recombinant envelope proteins conferred protection on challenge with low dose homologous virus (Choo et al., 1994).

Study of HVR evolution from single source infection demonstrated amino acid replacements limited to particular sets of amino acids which share similar properties (McAllister et al., 1998). This is hypothesised to be linked to host immune selection pressure. Affirming this in immuno-compromised individuals the sequence variation in the HVR is lower than that in immuno-competent individuals (Ray et al., 1999).

Neutralisation could prevent the virus interacting with the cell receptor, cross link the viral proteins preventing uncoating of the virus and therefore replication. Also antibody coated virus is more readily phagocytized by macrophages than free virus.

A neutralising of binding assay has been described where antibody from patients who resolved chronic HCV was tested for effect on reduction in binding of recombinant E2 to CD81 (Peleri et al, 1998). Epitopes which triggered responses which prevented binding in this assay were regions which were conserved between genotypes (Ishii et al., 1998).

Antibody antigen complexes play a major role in several complications and associated conditions of HCV infection (see section 1.7.3).

Emerging evidence indicates that cellular immunity mediated by both CD4+ and CD8+ T cells is more important in acute HCV. Individuals who recover from HCV infection appear to have a quantitatively more vigorous CD4+ proliferative responses against one or more HCV proteins compared to those individuals who develop chronic disease (Diepolder et al., 1997). The most commonly recognised CD4+ T cell epitopes are core amino acids 21-40, NS3 1251-1272, NS4 1767-1786 and NS4 1909-1929 (Lamonaca et al., 1999,

Diepolder et al., 1997). These sequences are conserved between HCV genotypes. HLA class II molecules from most MHC restrictions can bind these epitopes. HLA Class II allele (DRB1*01) is associated with spontaneous viral clearance (Barret et al., 1999).

The cytokine profile of T helper cells in resolved infection appears to be predominantly T_H1 , where as the T_H2 profile is associated with chronic infection (Tsai et al. 1997). Studies suggest that ribavirin may modulate the T helper Th_1/Th_2 subset balance in HCV infection (Hultgren et al., 1998) (Fang et al, 2000).

HCV-specific CTL can be readily isolated from the liver and PBMC of chronically infected individuals, and recognize multiple epitopes. Responses may play a critical role in recovery, and may persist for years after acute infection. CTL epitopes have also been identified in all viral proteins. In chimpanzees the onset of a multispecific intrahepatic CTL response correlated with viral clearance (Cooper et al., 1999). Uninfected individuals who have been multiply exposed to HCV exhibit peripheral blood HCV specific helper and CTL responses in the absence of Anti-HCV antibody or HCV RNA (Koziel et al., 1997; Scognamiglio et al., 1999). These CD8+ CTL may also cause tissue damage once chronic infection is established. Cytokines produced by these and other cells likely play a role in liver fibrosis. Destruction of HCV infected hepatocytes can occur by FasL/ Fas ligand binding triggering apoptosis, or Tumour Necrosis Factor α (TNF α) or perforin release from CTLs. One hypothesis is that the HCV-specific immune response is too weak to clear HCV from all infected hepatocytes once a persistent infection is established. MHC Expression of HCV antigens is low, 1%-10% of cells express antigen, 1-5% of mononuclear cells and an even smaller number of biliary epithelial or sinusoidal lining cells express HCV antigen (Nouri-Arai et al. 1995).

Reports indicate that individual HCV proteins may actively suppress the immune response of the host specifically, core, E2, and NS5A (Large et al., 1999). Numerous regulatory roles of HCV core that affect signal transduction, expression of viral and cellular genes, cell growth and proliferation are known. In addition, E2 and NS5A have been shown to interfere with the antiviral actions of interferon alpha (IFN α). IFN α is induced by the presence of double stranded DNA or RNA, as produced in the replication of viruses. IFN α acts by binding to a cell surface receptor and prevents translation of viral mRNA

without affecting cellular mRNA. The method of action involves induction of RNA activated protein kinase phosphorylation of translation initiation factor (eIF2 α) for cell protein synthesis, activation of 2,5-oligonucleotide synthetase which synthesizes adenine trinucleotide and adenine trinucleotide activates an endonuclease which degrades viral mRNA.

An E2 sequence of IFN resistant genotypes but not interferon sensitive genotype exhibits homology with the phosphorylation sites of IFN inducible protein kinase and translation initiation factor.

Regions of NS5 have been described as the interferon sensitivity-determining region. Many groups have disproved this association of sequence and response, however it is interesting that this protein binds and inhibits IFN inducible protein kinase (Gale et al., 1998).

NS3/4A has been shown to specifically inhibit a key immune system molecule, interferon regulatory factor-3 (IRF-3). IRF-3 orchestrates a range of antiviral responses. Without this master switch, antiviral responses never begin, and HCV can gain a foothold and persist in its host (Foy et al, 2003).

Studies suggest that circulating HCV has a half-life of only about 3hr indicating relatively efficient virus replication and release, at least in patients with high levels of viral load (Neumann et al., 1998). This dynamic process, capable of continuously generating viral variants, allows genetic variation to be an important strategy for the establishment and maintenance of persistent infection in order to adapt quickly to humoral or cellular.

1.9 Treatment

The end point of successful treatment is defined as sustained virological response (SVR). This is defined as the absence of detectable HCV in the serum by RT PCR at the end point of therapy and 24 weeks after the end of treatment. Treatment is with interferon α , or pegylated interferon α or a combination of an interferon and ribavirin. Interferon is a naturally occurring glycoprotein that is secreted by cells in response to viral infections. The interferon α works by binding to a membrane receptor and initiating a series of intracellular signalling events that leads to up-regulation of certain genes. This leads to target cell killing by lymphocytes and inhibition of virus replication in infected cells. Interferon injections are given subcutaneously, usually three times per week with dosage depending on the weight of the patient. Treatment is associated with several side effects including flu-like symptoms such as headaches, fever,

fatigue, loss of appetite, nausea, vomiting, and thinning of hair. In some instances, treatment with interferon can worsen the liver disease and be fatal. Pegylated interferon is interferon α -2b with polyethylene glycol side chains, which increase the half-life of the drug in the system. Ribavirin is an oral antiviral agent that is given twice a day. It is a synthetic guanosine nucleoside analogue. The way it works in the treatment of Hepatitis C is not completely understood, although it has been suggested that it works as a mutagen. Ribavirin treatment causes liver function tests to return to normal in some patients. Studies suggest that ribavirin may modulate the T helper Th₁/Th₂ subset balance in HCV infection rather than acting directly on the virus (Hultgren et al., 1998) (Fang et al, 2000). Combination treatment is associated with more side effects, such as severe anaemia, and depression. The combination of interferon α -2b and ribavirin gives SVR of 31-35% within 24 weeks of treatment and 38-43% after 48 weeks (McHutchison and Poynard, 1999). Use of pegylated interferon with ribavirin can increase the response rate to 65% (Manns et al., 2001). As with previous studies with interferon α (Jenkins et al., 1996) infection with genotype 1 resulted in lower rates of SVR (42%) than those with genotype 2 and 3 (82%) (Manns et al., 2001). It is unclear how to treat patients who do not respond to therapy after 48 weeks or less. However, it was demonstrated that up to 40% of non-responders developed evidence of a histological response on biopsy despite persistence of HCV RNA (Shiffman et al., 1999). Indicating a case for interferon or interferon plus ribavirin maintenance therapy.

1.9.1 New therapies

Several new therapies are being investigated for treatment of HCV. Research has focussed on compounds, which specifically target HCV. The HCV encoded serine protease, the HCV encoded helicase, HCV encoded RNA dependent RNA polymerase and the HCV RNA internal ribosome entry site have all been considered for potential therapy target sites.

Several groups have antisense oligonucleotide therapies in development with one compound ISIS 14803 (ISIS Pharmaceuticals, Carlsbad, CA., USA,) currently in human trials. In antisense therapies, synthetic stabilized DNA sequences bind highly specifically with target complementary RNA or DNA sequences. The formation of the hybrid induces host ribonuclease RNase H to degrade the heteroduplex, resulting in down regulation of gene expression. The

investigational agent ISIS 14803 targets HCV RNA at the core protein initiation codon. The antisense nucleotides can tolerate base pair mismatches, and therefore are not expected to be affected by sequence variation between different genotypes.

Similarly there is research in ribozyme therapy. The mode of action is similar to oligonucleotide therapy but instead of host enzyme degradation of duplex, with the ribozymes therapy, a small sequence of the ribozyme designated the catalytic core causes the RNA cleavage. Several groups have reported in-vitro inhibition of HCV gene expression using ribozyme against the 5' non-coding region. Ribozyme Pharmaceuticals (Boulder, CO., USA) have a compound, Hepazyme in human trials following encouraging results in animal experiments.

Another therapy which is in clinical trials is VX-497 a reversible non-competitive inhibitor of the enzyme inosine 5'-monophosphate dehydrogenase which catalyses a step in the synthesis of guanine nucleotides necessary for nucleic acids.

1.10 Detection

Prior to the isolation of the HCV genome non-A non-B, post transfusion hepatitis could only be diagnosed by testing the levels of the liver enzymes alanine and aspartate amino transferase in the serum. Now HCV infection can be detected by identifying one or more markers of infection. ELISA can screen blood for the presence of antibody to HCV and even very small amounts of viral RNA can be detected in blood or tissues using molecular biology.

1.10.1 Initial Serology

Three generations of EIA antibody testing have been developed since 1989. The EIA antibody is the main screening test for hepatitis C. The first-generation EIA antibody, which incorporated the c100-3 epitope from the non-structural NS4 region, was used until 1992, at which time it was replaced by a second-generation EIA (EIA-2). EIA-2 contains hepatitis C antigens from the viral core and from areas of the non-structural NS3 and NS4 regions (Barrera et al., 1995), the third-generation EIA that contains reconfigured core and NS3 antigens, and incorporated antigen from the NS5 region (Figure 1.7, Figure 1.8, Figure 1.9). EIA-3, with a sensitivity of 97%, offers slightly improved sensitivity over the 95% sensitivity seen with EIA-2 (Barrera et al., 1995).

There is no specific antibody associated with active infection; the presence of anti-HCV antibodies can be evidence of either a past, resolved infection or current infection. Haemodialysis or immunocompromised patients may have anti-HCV antibody titres below the threshold for detection by ELISA, resulting in false-negative serology (Thio et al., 2000)

Detection of antibodies to HCV utilizes indirect ELISA technology. Yeast or bacterially expressed recombinant HCV proteins or synthetic peptides are coated onto the solid phase of an ELISA plate, polystyrene bead or nitrocellulose strip. Anti-HCV antibodies in the sample to be tested bind to the captured antigen. The antibodies are visualized by adding anti-human immunoglobulin that is tagged to a marker. These markers are either radioactive compounds, or enzymes that catalyse chemiluminescent or colour change reactions in added reagents. The results can be determined by measuring radioactivity, visualised by colour change, measured spectroscopically from the colour absorption of the reaction or the energy produced by the chemiluminescence

1.10.2 Confirmatory Serology

Following a positive serology result a confirmation test is carried out. In high-risk populations the specificity of HCV screening is very high, however in lower risk populations, for example blood donor populations, the specificity is only about 60%. To resolve false positive results a confirmational test is carried out. The most common assay used is the recombinant Immunoblot (RIBA). This assay consists of nitrocellulose strips coated with separate bands of each of the HCV antigens, core, NS3, NS4 and NS5 (Figure 1.9). The positive screening result is confirmed only if a colour change is detected in two or more of these bands

In high-risk patients the screening result is more often confirmed by PCR. This way the HCV genotype or viral titre can also be determined. The use of nucleic acid screening in the US and Europe since July 1999 has negated the need for conformational assays.

Assay	Manufacturer	Technique	Epitopes	Genotype
Ortho 3.0 ELISA	Ortho-Clinical Diagnostics, Raritan, NJ	EIA microtitre plate	Core, NS3, NS4 ,NS5	1
Vitros anti-VHC	Ortho-Clinical Diagnostics, Raritan, NJ	EIA automated on Vitros ECI device	Core, NS3, NS4 ,NS5	1
Murex VK48	Murex Diagnostics, Dartford UK	EIA microtitre plate	Core, NS3, NS4 ,NS5	1
Abbott HCV EIA 3.0	Abbott Diagnostics, Chicago, IL	ELISA microbeads	Core, NS3, NS4 ,NS5	1
IMx HCV3.0	Abbott Diagnostics, Chicago, IL	EIA automated on IMx device	Core, NS3, NS4 ,NS5	1
AxSYM HCV 3.0	Abbott Diagnostics, Chicago, IL	EIA automated on AxSYM device	Core, NS3, NS4 ,NS5	1
Monolisa anti-HCV plus version2	Bio-Rad, Coquette	EIA microtitre plate	Core, NS3, NS4	1
Access HCV Ab Plus	Bio-Rad, Coquette	EIA automated on Access device	Core, NS3, NS4	1
Innotest HCV ab IV	Innogenetics, Ghent, Belgium	EIA microtitre plate	Core, NS3, NS4 ,NS5	1, 2, 3a

Figure 1.7 The components of a selection of the most common commercially available Diagnostic EIA assays for detection of Anti-HCV antibody.

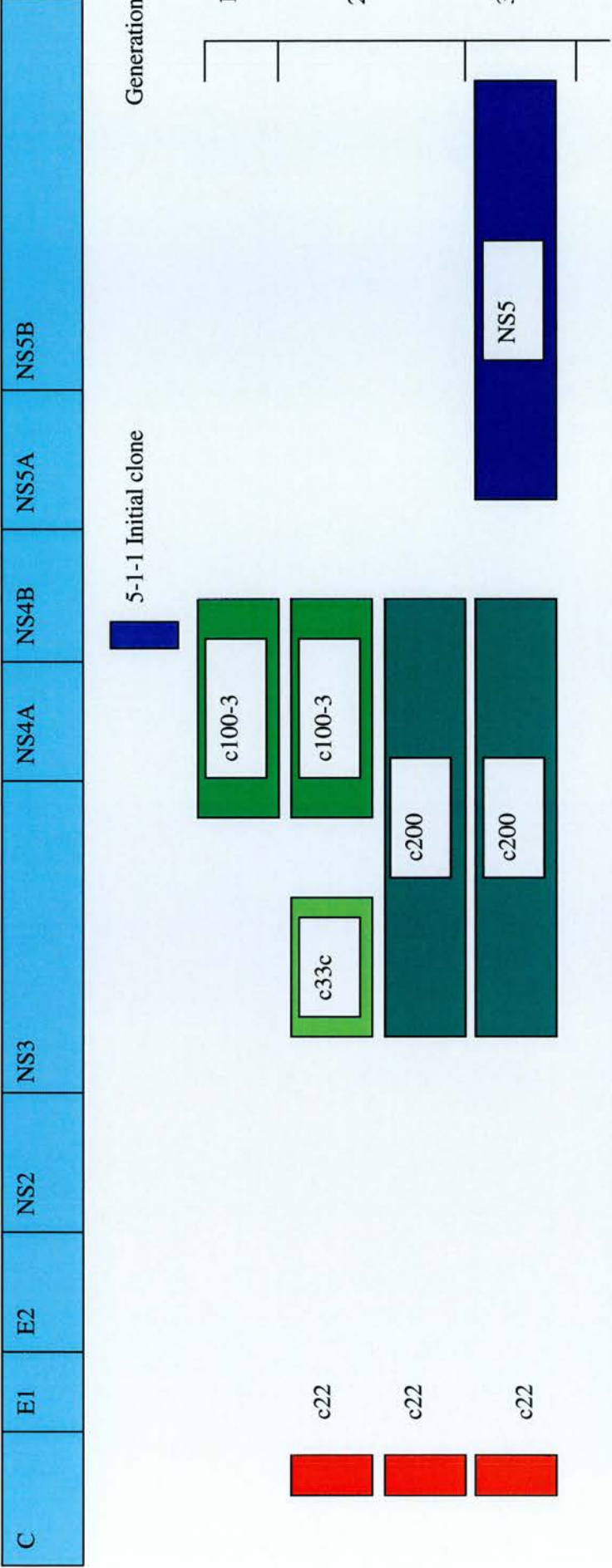


Figure 1.8 Antigenic regions used in commercial screening assays

ANTIGENS USED FOR ANTI-HCV SCREENING

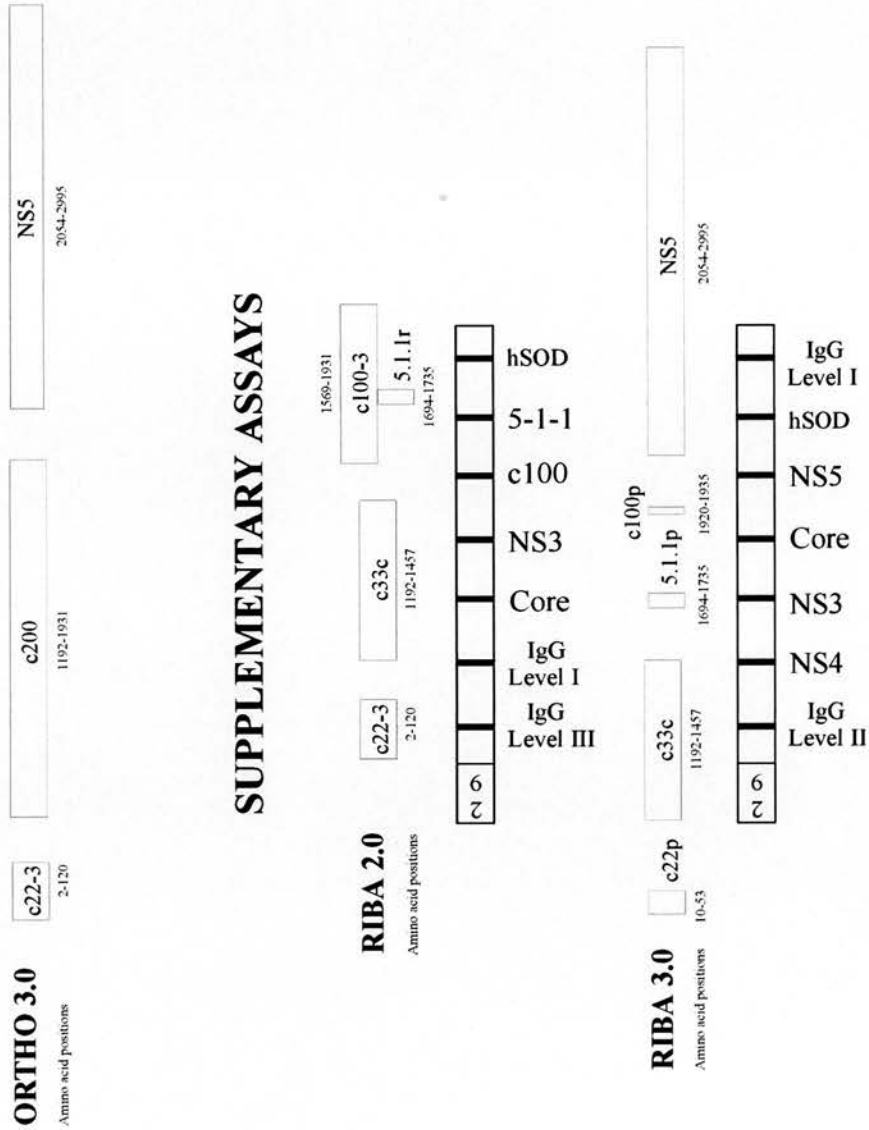


Figure 1.9 Construction of the confirmatory RIBA 2.0 and 3.0 Assays

1.10.3 Determination of clearance/ chronic carriage

The demonstration of hepatitis C viral particles in blood confirms the diagnosis of hepatitis C infection. Methods used to detect hepatitis C viral RNA are target amplification and RNA amplification

RNA amplification assays such as the polymerase chain reaction (PCR) rely on sequence-specific primers and a heat-stable DNA polymerase to generate a large number of copies of a portion of the viral genome. Signal amplification, as used in bDNA assays, uses a series of hybridisation reactions between probes specific for several regions of the target molecule and subsequent hybridisation to a DNA amplifier (Gretch et al., 1995).

The specificity of the current RNA detection test is 98-99%. The lower limit of the assay cut-off is 50IU/ ml. The IU is defined against the WHO HCV RNA standard.

1.10.4 Genotyping

Linear antigenic determinates were identified in core and NS4 regions which were known to differ among the HCV genotypes (Tsukiyama Kohara et al., 1993), (Simmonds et al., 1993b). Peptide ELISAs were constructed which allowed identification of the type-specific response to the genotype of infection. Serotype 1 and 2 were detected with core (Mondelli et al., 1994) or NS4 antigens (Tsukiyama Kohara et al., 1993), (Tanaka et al., 1994). Use of a solution of competing peptides allowed the determination of serotype 1, 2 or 3 (Simmonds et al., 1993b) or serotypes 1-6 with NS4 branched peptides (Bhattacharjee et al., 1995). Murex Diagnostics Ltd. marketed the ELISA constructed with genotype 1-6 branched peptides. Chiron Diagnostics marketed a confirmation RIBA incorporating genotypes 1, 2 and 3 NS4 and 1 and 2 core antigens (Digit et al., 1995).

1.10.5 Viral Load Testing

The World Health Organisation has established an international standard for universal standardization of HCV RNA quantification units (Sultana et al., 1999). An IU for HCV RNA has been arbitrarily defined using this standard, it represents amount of RNA in a sample rather than copies per ml. All quantitative assay now report in these units.

The HCV RNA level can be quantified by means of target amplification techniques; polymerase chain reaction or transcription-mediated amplification, or signal amplification ("branched DNA" assay).

1.10.6 HCV core antigen

Total core antigen can be detected and quantified by means of an EIA assay, Ortho-Clinical Diagnostics). It has been shown that HCV core antigen titers (in pg/ml) correlate with HCV RNA levels (Bouvier-Alias et al. 2002). It can be used as a surrogate marker of HCV replication. It has been estimated that 1 pg of total core protein antigen per ml is equivalent to approximately 8000 IU. It is detected 1-2 days later during the acute infection period than HCV RNA by current assays and the limit of detection is 20000 IU/ml which limits its use in the clinical setting.

1.11 Aims of the project

1.11.1 Investigation of HCV genotype-specific antibodies

Extensive genomic sequence variation (30-33% at nucleotide level) exists between the different genotypes of HCV. The extent of variation differs among genomic regions. Structural and functional constraints are thought to influence the extent of variation in certain areas. The first aim of this project was to investigate whether the immunogenic regions and epitopes are influenced by genetic variation and to investigate the implications this has for anti-HCV antibody screening.

Commercial screening assays are based on genotype 1 sequence, as this is the most prevalent genotype in the U.S.A. and Europe. The other genotypes of HCV are prevalent in other areas of the world. Areas of the developing world have extremely high, and often increasing, prevalence of HCV. It is important to prevent further transmission; therefore, anti-HCV detection in blood donations must be sensitive and accurate.



Reliable rapid screening for HCV antibodies in donated blood ultimately requires a single ELISA to detect infection with a variety of genotypes. The antigen used in such an assay must therefore have epitopes that react with all genotypes. Ideally, this would detect antibodies in all infected individuals regardless of the infecting genotype.

Through experiments on the reactivity of sera from genotype 1 and non genotype 1 infections, using genotype 1 and non type 1 antigens, I aimed to determine the extent of genotype-specific reactivity and cross reactivity. The overall aim is to ascertain which combination of antigens best detects infection with all genotypes.

1.11.2 Investigation of level of detection of heterologous HCV genotype infection

I aim to measure reactivity, by comparing to a standard, to assess if the detected reactivity in heterologous genotype infection is adversely affected by the type-specific component. I also aim to test blood donations from areas of non type 1 prevalence to investigate the serological detection rate compared to the molecular biological detection rate of infectious sera.

1.11.3 To investigate if the use of heterologous antigens can detect infections undetectable by current serological methods, but detected by molecular biology

I aim to use single antigens from heterologous genotypes and a heterologous genotype screening assay to screen samples from risk groups in areas where genotypes other than 1 are prevalent to try to detect additional HCV infections. I also aim to investigate samples from blood donors and patients whose HCV infections have been discovered by molecular biological techniques and is repeatedly undetectable by third generation assays.

1.11.4 To investigate if use of heterologous HCV genotype antigens can resolve confirmational assay indeterminate samples

In areas where HCV prevalence is low, definitive evidence of serological reactivity to HCV depends on reactivity to at least two independent antigens as a small proportion of individuals (approximately 0.5%) may show non-specific serological reactivity to HCV antigens. A Chiron RIBA immunoblot assay is often used as such a confirmatory test. Samples showing non-specific reactivity usually react weakly with only one antigen and this is classed as an indeterminate. However, a small proportion of indeterminate samples may be

found in individuals infected with HCV but who show a highly restricted serological response to infection. One reason for non-reaction to antigens in the confirmation test is antigenic variation, particularly amongst the non-structural proteins, NS3, NS4 and NS5. It is therefore possible that inclusion of antigens from other genotypes may help in the identification of the anti-HCV positive samples. An indeterminate sample often will be sent for PCR testing for confirmation. I aim to investigate if a heterologous antigen assay could confirm these infections.

1.11.5 Investigation to determine if heterologous HCV antigen assays can increase sensitivity of detection in the “window phase”

The window phase is the time between infection and detection of infection. This is the most critical time for blood donation centres as infected donations may pass undetected. At this time, PCR positivity and elevated alanine transferase levels are the only indications of infection. I aimed to test prospectively followed patients, acutely infected with non-genotype 1 variants of HCV. This would investigate whether serological-testing with antigens from the infecting genotype were more effective, at an earlier time point, than with genotype 1 antigen.

Chapter 2

2 Materials and Methods

2.1 Polymerase chain reaction

Polymerase chain reaction (PCR) was used for amplifying large quantities of deoxyribonucleic acid (DNA) from small concentrations of the starting product. This process was extremely sensitive and was capable of isolating the required sequence by copying it several thousand fold in a short time. The reagents required for PCR were: a DNA template or a complementary DNA (cDNA) template (constructed from a ribonucleic acid (RNA) sequence by a reverse transcriptase reaction), deoxynucleotide triphosphate (dNTPs), two oligonucleotide primers one sense and the other antisense, reaction buffer containing magnesium and a thermostable DNA polymerase. The reactions took place in a thermocycler that controlled the temperature and length of time at any given temperature.

Each PCR reaction consisted of 20-30 cycles and each cycle consisted of three stages: denaturing, annealing and extension. In the first stage the template was denatured by heating to around 94 °C for 20 seconds which destabilised the base pairing interactions that held the nucleic acid strands together. The second stage cooled the temperature to between 40-60 °C, depending on the nature of the primer, for 21 seconds. This allowed the primers to anneal to the single strands. The third stage extension increased the temperature to 72 °C, which allowed the thermostable DNA polymerase enzyme to utilise the added dNTPs to synthesise new double stranded material. This was followed by the denaturing step of the second cycle.

Nested or secondary PCR reactions were carried out to further select the desired sequence and to increase the amount of product. Primers were designed which corresponded with sequence downstream from the site of the primary primer sites and the PCR was performed under the same conditions, but a smaller volume of reactants was used and a smaller sized amplicon produced. Amplification was logarithmic, with each cycle producing double the start product amount, but was limited in the final stages by the concentration of reactants.

The DNA polymerase enzymes used were heat stable as they were obtained from organisms which could tolerate high temperatures, for example, *Thermus aquaticus* from which the Taq enzyme was isolated and *Thermus flavus* the source of the Tfl enzyme.

2.1.1 Detection of viral RNA by using PCR amplification

2.1.1.1 Preparation of serum sample

Blood specimens were obtained by venepuncture and collected in sample tubes. These were transported to the laboratory, where they were centrifuged at 5 000 g to pellet erythrocytes. The upper serum layer was gently removed by Pasteur (Alpha, Eastleigh, UK) and collected in 1.5 ml Eppendorf tubes (Anachem, Luton, UK), which were immediately sent for analysis or stored at -20 °C refrigeration.

Plasma samples were obtained from blood donations, where the blood drawn was treated with buffered sodium citrate anticoagulant and the red cells were removed by fractionation.

2.1.1.2 Extraction of nucleic acid from serum using polyadenylic acid as a carrier.

An aliquot of 100 µl serum was mixed with 400 µl extraction mix (Appendix 1), which had been pre-incubated in a water bath at 37 °C which inactivated RNases, then incubated at 37 °C in a water bath for two hours. The extraction mix contained: sodium dodecyl sulphate (SDS), which lysed cells present; proteinase K, which digested proteins in the sample for efficient phenol chloroform removal; and polyadenylic acid (poly A), a large carrier molecule that helps precipitate the nucleic acids. The mixture was agitated with 450 µl phenol (Rathburn Chemicals, Walkerburn, UK) for 15 minutes then centrifuged at 12 000 g for 15 minutes. The resulting supernatant was removed to a 1.5 ml Eppendorf tube containing 450 µl of 50:1 chloroform:isoamylalcohol mixture (BDH, Lutterworth, UK), which was, again, agitated for 15 minutes, then centrifuged at 12 000 g for 15 minutes. The final supernatant was removed to another 1.5 ml Eppendorf tube where the nucleic acids were precipitated by the addition of 40 µl 3M sodium acetate pH5.2 (BDH) and 600 µl 100% ethanol (BDH) and refrigerated at -20 °C for a minimum of 6 hours. The precipitated nucleic acids were collected as pellets in the bottom of the tube spinning at 100 000 g in a centrifuge, previously cooled to 0 °C, for 30 minutes. The

supernatant was poured off and the pellets were washed with 500 µl chilled 80% ethanol and centrifuged for 5 minutes. The excess ethanol was gently removed with a pipette and the pellet was dried on a hot block at 37 °C for 5 minutes to evaporate the residual ethanol. The pellet was dissolved in 20 µl of diethyl pyrocarbonate (DEPC) (Sigma, Poole, Dorset) treated water by slowly pipetting the liquid up and down. The dissolved nucleic acid was either used immediately for a reverse transcription reaction or stored at -40 °C refrigeration.

2.1.1.3 Reverse transcription reaction

Before PCR can be used, single stranded nucleic acid must be converted to double stranded. The enzyme RNasin was used to inhibit any ribonucleases from degrading the RNA and HCV RNA was converted to cDNA by the enzyme reverse transcriptase. For this reaction, an aliquot of 5 µl of nucleic acid, dissolved in DEPC treated water, was incubated on a hot block at 42 °C for 30 minutes with: 10 units reverse transcriptase from avian myeloblast virus (AMV RT), 10 units RNasin (both Promega, Southampton, UK), 1X RT buffer [50 mM Tris (hydroxymethyl) methylamine hydrochloride (Tris-HCl) pH8 (BDH), 5 mM magnesium chloride (MgCl₂) (BDH), 5 mM dithiothreitol (DDT) (BDH), 50 mM potassium chloride (KCl) (BDH) and 0.5 mg/ml bovine serum albumin (BSA)] (Promega), 1mM external reverse primer (Figure 2.1), 20% dimethylsulphoxide (DMSO) (Sigma), 600 µM each of deoxyribonucleotide triphosphates dNTPs (deoxyriboadenosine triphosphate dATP, deoxyribothymine triphosphate dTTP, deoxyribocytidine triphosphate dCTP, deoxyriboguanine triphosphate dGTP), and 4.5 µl DEPC treated water. The cDNA was either used immediately for PCR, or placed in -40 °C storage.

2.1.1.4 Polymerase chain reaction primary reaction

The cDNA from the reverse transcription stage was amplified by 20-30 cycles in a PCR machine, depending on the region to be amplified and the primers used. A volume of 5 µl of the cDNA was dispensed into a PCR tube containing a final concentration of: 1 unit Taq enzyme (Promega), 1X Taq buffer (1.5 mM MgCl₂, 10 mM KCl, 2 mM Tris-HCl pH8, 0.01 mM ethylene diamine tetraacetic acid (EDTA), 0.1 mM DDT, 5% glycerol, 0.05% Nonidet P-40 and 0.05% Tween 20)(Promega), 33 µM each dNTPs, 1 µM external sense primer (Table 2.1), 1 µM external antisense primer (Figure 2.1) and DEPC treated

water to 50 µl. Gentle pipetting up and down mixed the contents, before 2 drops of liquid paraffin (William Ransom, Hitchin, UK) was added which prevented the solution from evaporating. The PCR tube was placed on a thermocycler (Genie, Techne Anachem, Luton, UK) and the programme set. For the 5' non coding region (NCR), this was: denaturing at 94 °C for 18 seconds, annealing at 50 °C for 21 seconds and extension at 72 °C for 90 seconds for 25 cycles, followed by a final extension at 72 °C for 6 minutes to remove any single stranded product. Different primers and PCR conditions were used for amplification of each region (Figure 2.1, Table 2.1).

2.1.1.5 Nested or secondary PCR reaction

To increase the specificity of the amplification and increase the amount of DNA produced, a secondary, or nested, PCR was carried out. This consisted of the same conditions as the above primary reaction, but the internal sense and anti sense primers were used, instead of the external ones and the reaction was carried out in a 20 µl volume.

2.1.1.6 Agarose gel electrophoresis and ultra-violet radiation detection of nucleic acids

20 µl of secondary PCR product was subjected to electrophoresis on a 2% agarose gel (Flowgen, Litchfield, UK), containing 0.5 mg/ ml ethidium bromide (Sigma). The gel was placed in a gel tank in 1X Tris Borate EDTA (TBE) solution (Appendix 1) and the voltage differential set at 150 Volts for 10 minutes. The amplified nucleic acid was visualised by fluorescence under ultra-violet light. Resulting bands in the gel were photographed by a Polaroid camera (HayWest X-ray, Edinburgh, UK).

2.1.1.7 Controls

The success of the PCR could be determined by the results of the control sample that was always included with any batch of samples. The control sample was of approximate RNA titre 1×10^4 copies/ ml and was titrated in negative sera in dilutions, ranging from 1:10 to 1:1 000 000 and 100 µl of these extracted. A batch of PCR reactions was considered successful when 1:10-1:10 000 dilutions were positive. A negative control sample was also used in every batch to detect false positives arising from contamination. A size marker was used during electrophoresis on some occasions to check the size of the amplified product.

No.	Region	Sequence ^a
209	NCR	GGT GCA CGG TCT ACG AGA CCT
939	NCR	GTG TGA GGA ACT ACT GTC TT
211	NCR	CAC TCT CGA GCA CCC TAT CAG GCA GT
940	NCR	TTC ACG CAG AAA GCG TCT AG
954	CORE	ACT GCC TGA TAG GGT GCT TGC GAG
410	CORE	GAG CGG AAT GTA CCC CAT GAG GTC GGC
236	CORE	GGT AGA CCG <u>GAT CC</u> ^b C ATG AGC ACG AAT CCT AAA
597	CORE	GAA AGA <u>AGC TTA</u> GAC GAG CGG AAT GTA CCC
751	NS3	TTY CGG GCN GCY GTG TGC ACC
753	NS3	GCG TCA TAG CAC TCA CAG AGG AC
594	NS3	ACC <u>RCT GCA</u> GAY CCY AAC ATN GAG GAR GT
593	NS3	CAC <u>AAG CTT</u> ARC AGT AGT CKA TSA CNG ART C
750	NS3	TGG TYA CNN GNN AYG CYG ATG TCA TYC C
007	NS3	ACC TCG AGT ATC CCA CTG ATG AAG TTC CAC AT
991	NS5	TGG CNA GCT CNT CNG CNA GCC A
993	NS5	GGC TCC CCC TCA AGG GRG GCA TRG A
3155	NS5	<u>GGA TCC</u> AAY ATN ACY CGY GTR GAG TCW SA
3156	NS5	<u>AAG CTT</u> GTG GTG GYA AGG CAC AYC C
PRIMERS USED FOR AMPLIFICATION OF SEQUENCE FROM pTag		
VECTOR		
8819	pTag	GCT ATG ACC ATG ATT ACG CCA A
9130	pTag	ACA CGT GTG GTC TAG AGC

Figure 2.1 Primers used for amplification of NCR, CORE, and NS3 AND NS5 regions.

^a IUPAC ambiguity codes (Y:C/T; R:A/G; N:G/C/A/T; K:G/T; S:C/G; W:A/T)

^b Cleavage sites for restriction enzymes underlined: CTGCAG PstI, AAGCTT Hind III, GGATCC BamHI
939, 940 (Okamoto et al., 1990) 209,211 (Garson et al., 1990)

410, 954 (Mellor et al., 1995)

236, 597 Murex Biotech.

751, 753, 593, 750, 007, 991, 993, 3155, 3156 (Molecular Virology lab)

8819, 9130 pTag vector kit.

Region	RT ^a	1°PCR ^b	1PCR ^c	2PCR ^b	2PCR ^c	no cycles	size (bases)	Programme ^d
NCR	209	939	209	940	211	(-29 to -54)	225	1
Core	410	954	410	236	597	(432 to 403)	442	2
NS3 Type 3	753	751	753	594	593	(4372 to 4341)	276	2
NS3 Type 4	007	750	007	594	593	(4372 to 4341)	276	2
NS5 Type 3	993	991	993	3155	3156	(6979 to 6954)	228	2
NS5 Type 4	993	991	993	3155	3156	(6979 to 6954)	228	2

^a carried out at 42°C for 30 minutes

^b sense

^c antisense

^d Programme 1 denaturing at 94 °C for 18 seconds, annealing at 50 °C for 21 seconds, extension at 72 °C for 90 seconds, with a final extension at 72°C for 6 minutes after 25 cycles. Programme 2 differed from programme 1 by annealing at 45 °C for 21 seconds.

Table 2.1 Coordinates of primers and size of amplicon produced and conditions required

2.1.1.8 Nucleic acid extraction using glycogen as carrier

The Access kit (Promega) method of extraction differed from the direct extraction above by the use of glycogen as the carrier molecule to extract RNA. The AMV-RT reaction and the primary PCR occur in the same reaction vessel and the enzyme Tfl was used in preference to Taq. The Access protocol required 250 µl of plasma or serum, mixed with 250 µl of Access Extraction Mix incubated for 1.5 hours at 37 °C in a water bath. Phenol and chloroform:isoamylalcohol (50:1) extractions were performed as above. The supernatant of the chloroform:isoamylalcohol extraction was dispensed into a 1.5 ml Eppendorf tube with 1 µl of glycogen, 40 µl 3 M NaCO₂CH₃ pH 5.2 and 800 µl of 100% ethanol. This was refrigerated at -20 °C for a minimum of 6 hours, before nucleic acids were pelleted by 12000 g for 15 minutes in a centrifuge, and then washed with 80% ethanol to remove any soluble contamination. The collected nucleic acids were suspended in 20 µl DEPC water. The combined reverse transcription and primary PCR reaction utilised 5 µl RNA mixed with final concentration of 5 units AMV-RT, 5 units Tfl DNA polymerase, 1 mM magnesium sulphate (MgSO₄), 1 µM sense primer (Figure 2.1), 1 µM antisense primer (Figure 2.1), 0.2 mM dNTP, 1 X AMV-RT/Tfl reaction buffer and 23 µl DEPC water. The PCR tube contents were overlaid with liquid paraffin and placed on a thermocycler set for 1 cycle at 48 °C for 45 minutes, the conditions for the RT reaction; 1 cycle at 94 °C for 2 minutes, to inactivate the AMV-RT enzyme; followed by 40 cycles of denaturing at 94 °C for 30 seconds; annealing at 60 °C for 60 seconds and extension at 68 °C for 120 seconds. Secondary PCR was carried out using the same protocol as section 2.1.1.5 above.

2.1.2 Restriction fragment length polymorphism

Restriction fragment length polymorphism (RFLP) was used to distinguish between genotypes and subtypes of HCV (McOmish et al., 1993), (McOmish et al., 1994) and (Davidson et al., 1995). RFLP is based upon the phenomenon whereby two almost identical DNA or cDNA sequences are distinguished from each other by the difference in size and/or number of product fragments produced when cut by restriction endonucleases.

These variations, caused by small differences in nucleotide sequence in the palindromic sites the restriction enzymes, recognise lead to a distinction between excisions in the sequences, visualised by distinctly different product band patterns on metaphor agarose gel electrophoresis.

2.1.2.1 RFLP of 5'NCR – Genotypes

Genotypes of HCV were determined as described by Mc Omish and colleagues (McOmish et al., 1994). A 5'NCR PCR fragment amplified with primers 939 and 209, followed by a secondary PCR reaction, performed using the same reagent volumes as a primary reaction using 940 and 211, was pipetted into two aliquots of 20 µl. One aliquot was digested with 1 unit Rsa I and 1 unit Hae III in 3 µl buffer C (10 mM Tris-HCl, 10 mM MgCl₂ 50 mM NaCl, pH 7.9) (all Promega). The other aliquot was digested with 1 unit MvaI and 1 unit HinfI in 3 µl buffer B (6 mM Tris-HCL, 6 mM MgCl₂, 50 mM NaCl, pH 7.5) (also all Promega). Both were left at 37 °C for between 6 and 24 hours. The product was visualised by electrophoresis on a 4% metaphor agarose (Flowgen) gel containing 0.05 mg/ ml ethidium bromide for 60 minutes at 150 Volts in 1X TBE buffer (Appendix 1). Different patterns were representative of different genotypes (Figure 2.2).

2.1.2.2 RFLP of 5'NCR - Subtypes

Subtype 1a could be distinguished from subtype 1b by restriction digest with the BstU1 enzyme. Subtypes a and b of genotypes 2 and 3 could be differentiated with ScrF1 enzyme digest.

Primary and secondary PCR reactions with primers 209, 939 and 211, 940 were carried out as described previously. An aliquot of 20 µl PCR product was digested with either 5 units of BstU1, 0.3 µl 1M MgCl₂, 0.375 µl 0.1 DDT and 3.5 µl distilled water at 60 °C for a minimum of 4 hours, or 5 units of ScrF1 in 3 µl of 10 x ScrF1 buffer at 37 °C for 6 hours. The subtype could be identified by the pattern of digested fragments (Figure 2.3) of PCR product produced following electrophoresis on a 4% metaphor agarose (Flowgen) gel containing 0.05 mg/ ml ethidium bromide for 60 minutes at 150 Volts in 1X TBE buffer (Appendix 1)

Size of fragments (kb)					HCV genotype				
a	44	58	114/5		9	26		1, 4	
b	102		114/5		9	26		1, 4, 5	
c	44	12	58	56	9	26		2	
d	44	12	114		9	26		2	
e	56	46	114		9	26		2	
f	33	69	114		9	26		3	
g	33	23	114		9	26		3	
h	44	12	117		9	26		6	
Genotypes Hae III / Rsa I Digests Ladders									
A	53	63	41	94				1	
B	53	63	44	94				6	
C	53	56	142/3					3, 4	
D	53	198						2, 5	
Genotypes Mva I /Hinf I Digest Ladders									

Figure 2.2 Analysing the combination of the HaeIII/RsaI and MvaI/HinfI

DNA band fragment patterns, the genotype of HCV can be determined. Extracted from (McOmish et al., 1994) with permission from the authors

Size of fragments (kb)				Subtypes	
A	209			42	1a,
B	179	30		42	1b

Subtypes BstUI1 digest ladder genotype 1

Size of fragments (kb)				Subtypes		
D	53	15	48	41	94	2a, 2c
E	53	15	183			2b
F	53	15	48	135		2b

ScrF1 digest ladder genotype 2

Size of fragments (kb)				Subtypes			
G	53	15	57	126		3a	
A	53	15	48	9	32	94	3b

ScrF1 digest ladder genotype 3

Figure 2.3 RFLP fragments after digestions with BstUI and ScrF1 enzymes, allowing distinction between a and b subtypes.

2.2 Cloning

Cloning was the technique used to manipulate PCR fragments into vectors for sequencing, transport and recombinant protein expression. These fragments were inserted into the plasmid cloning vectors by one of two methods: use of a thymidine over-hang vector to complement the poly-adenine present at the end of fresh PCR product; or restriction enzyme site incorporation into the secondary primer sequence. The first method described was used for sequencing and transport. The restriction sites within the secondary primers were utilised by the research facility at Murex Biotech Ltd to excise the required DNA sequence and insert it in the correct orientation into the protein expression systems. The clones constructed were transformed into *Escherichia coli* (*E.coli*) cell cultures and multiple copy plasmid DNA and recombinant protein produced. Stock cell preparations of these clones were stored in glycerol at -70 °C for future use and production.

2.2.1 pTag Vector - LigATor kit use of A-T pairing for insertion into vector

The LigATor cloning (R and D systems, Abbingdon, UK) system was used to sub-clone PCR fragments for sequencing and for transport to Murex Diagnostics where cloning into expression systems took place.

The pTag vector contained thymidine base overhangs at the insertion site, which facilitated the cloning of fresh PCR product, which contains complementary adenine bases, into these sites. The plasmid vector contains the LacZ α gene that was used as a reporter gene. This allowed selection of white colonies of insert containing recombinants from blue colonies of recombinants that do not contain insert, when grown on 5-bromo-4-chloro-3-indolyl- β -D galactoside (X-gal) containing media. The plasmid also contained genes for resistance to ampicillin, tetracycline and kanamycin that were used to select only plasmid containing colonies. The kanamycin resistance reporter gene facility was not utilised. Tetracycline prevented the loss of the F' plasmid containing LacZ M15 in the competent cells which would cause the loss of functional β -galactosidase production, resulting in all-white colonies, regardless of plasmid content.

2.2.2 PCR preparation

PCR product fresh from a secondary PCR reaction was treated by shaking with an equal volume of 24:1 chloroform:isoamylalcohol to remove traces of paraffin oil and nucleases. This was centrifuged and the aqueous layer removed for use. A phenol:chloroform extraction and precipitation with ethanol and sodium acetate was carried out if the DNA concentration was too dilute. Alternatively, the required band of PCR product was excised from a 0.8% low melting point Agarose (Flowgen) gel following electrophoresis for 10 minutes at 150 Volts in 1X TBE (Appendix 1). The excised band was placed in a 1.5 ml Eppendorf tube, diluted 1:1 in Tris-EDTA (TE) solution (Appendix 1) and the gel dissolved at 65 °C in a water bath. This solution was extracted twice with an equal volume of phenol to remove the agarose before a 1:1 phenol:chloroform extraction, followed by nucleic acid precipitation at -20 °C for 2 hours with 1/10 volume NaCO_2CH_3 and 2 volumes 100% ethanol.

The nucleic acids were collected by a 15-minute spin at 10000 g in a centrifuge and then diluted in 10 μl distilled water.

2.2.3 Ligation Reaction

The ligation reaction was set up in a 1.5 ml Eppendorf tube with 1 μl of 50 ng/ μl (0.03 pmol) pTAg vector, 1 μl 10 X ligase buffer (200 mM Tris-HCl pH7.6, 50 mM MgCl_2), 0.5 μl 100 mM DDT, 0.5 μl 10 mM ATP, 2 μl of fresh PCR product (approx 0.2 pmol) and 4.5 μl of sterile water. The ligation was catalysed by the addition of 0.5 μl T4 ligase (2-3 Weiss Units). This was gently mixed with a pipette then incubated at 16 °C overnight.

A ligation control reaction containing 2 μl (5 ng) of a 50 base pair control insert (instead of PCR product) and a self ligation control reaction using 2 μl of water (instead of PCR product or control insert) was also carried out.

2.2.4 Transformation Reaction

Aliquots of frozen competent cells, genotype *endA1 hsdR17(rk₁₂⁻m_{k12}⁺) supE44thi-1 recA1 gyrA96 relA1 lac[F' proA⁺B⁺ lacI M15::Tn10(Tc^R)]*, were removed from -40 °C storage and allowed to defrost slowly on ice. A 1.5 ml Eppendorf tube for each reaction was cooled in the ice and 20 μl of cells were transferred to each tube. After the cells were cooled, 1 μl of the ligation reaction was added and gently mixed. The mixture was left for 30 minutes on ice before a 40 second "heat shock", which transformed the plasmid into the

cells, was carried out by placing the tube in a 42 °C water bath for 40 seconds. The tubes were then placed on ice for a further 2 minutes, before 80 µl of SOC medium (Appendix 1) was added and the tubes transferred to a rotary shaker that was incubated at 37 °C for 1 hour.

In the test plasmid control 1 µl of test plasmid was added to the cells in the place of ligation reaction. After the incubation, 50 µl of the transformants (20 µl of test plasmid control) were spread on Luria-Bertani agar (Appendix 1), supplemented with 50 µg/ ml ampicillin and 15 µg/ ml tetracycline and a coating of 100 µl isopropyl-thiogalactoside (IPTG) and 20 µl 50% X-gal and incubated at 37 °C for 16 hours. White colonies were picked out using sterile technique and tested by PCR for the presence of the insert, using either pTAg primers (8819, 9130) or the initial nested PCR primers (Figure 2.1).

The PCR products were subjected to electrophoresis for 15 minutes at 150 volts, on a 2% agarose gel containing 15 µl 0.05 mg/ ml ethidium bromide in 1 X TBE (Appendix 1) solution. The colonies that contained inserts of the required sizes were grown as mini preps.

2.2.5 Mini-Prep for Extraction of DNA

Colonies known to contain the required insert were picked with a sterile toothpick and placed in 3 ml of LB broth (Appendix 1) in a 10 ml centrifuge tube and incubated in the rotary shaker at 37 °C for 16 hours. The culture was then decanted into 1.5 ml Eppendorf tubes and spun at 12 000 g for 15 seconds: this pelleted the cells to the bottom. The supernatant was removed and the cells resuspended in 100 µl glucose-Tris-EDTA-lysozyme solution (GTE-lysozyme) (Appendix 1), 200 µl of freshly prepared sodium hydroxide SDS solution (Appendix 1) and placed on ice for 5 minutes. A 150 µl volume of potassium hydroxide-glacial acetic acid solution (Appendix 1) was then added and the tubes were vigorously shaken. This mixture was left on ice for 5 minutes. GTE-lysozyme broke down the cellular components. NaOH-SDS denatured the cellular DNA: plasmid DNA is super coiled and can renature. Potassium hydroxide-glacial acetic acid aggregated the denatured DNA, which was pelleted when spun for 8 minutes at 12 000 g in a centrifuge. The supernatant was removed to another 1.5 ml Eppendorf tube containing 290 µl of isopropanol, which was left to incubate at room temperature for 15 minutes, before being spun for 10 minutes at 12 000 g.

After the supernatant was discarded, the pellet was washed with 500 μ l 70% ethanol before it was dried and resuspended in 40 μ l Tris EDTA solution containing RNase. This was stored at -40 °C for later use or used for sequencing reactions.

2.3 Sequencing

Sequencing was carried out using the Sanger method. This technique utilized 2',3'-dideoxynucleotide triphosphates (ddNTPs), molecules that differed from deoxynucleotides by a hydrogen atom attached to the 3' carbon instead of an OH group. These molecules terminated DNA chain elongation because they could not form a phosphodiester bond with another deoxynucleotide. Sequencing was carried out on single stranded DNA, therefore, double stranded DNA was denatured with NaOH. For each sequence in question four Sanger reactions were carried out. Each of the reactions contained one of the dideoxynucleotides with the four deoxynucleotides, for example, ddATP with dATP, dTTP, dCTP and dGTP. A Sanger sequencing reaction consisted of the following: a strand to be sequenced (one of the single strands which was denatured using NaOH), DNA primer, radioactively labelled dATP, a mixture of a particular ddNTP and the four dNTP. The concentration of ddNTP was 1% of the concentration of dNTPs. After DNA polymerase was added, the polymerisation took place and terminated whenever a ddNTP was incorporated into the growing strand. As the ddNTP was only 1% of the total concentration of dNTP, a whole series of labelled strands resulted with the lengths of these strands dependent on the location of the base relative to the 5' end. This reaction was performed four times using a different ddNTP for each reaction. When these reactions were complete, a polyacrylamide gel electrophoresis (PAGE) was performed. Each reaction was loaded into one lane for four lanes. The gel was transferred to filter paper and autoradiography performed. The DNA sequence was deciphered by noting the order of migration of each of the bands on the film.

2.3.1 DNA preparation for sequencing reactions

The DNA strands obtained from a mini-prep were denatured by taking an aliquot of 20 µl resuspended DNA, which was incubated at 37 °C, with 2 µl of 2 M NaOH/ 2 mM EDTA solution for 30 minutes. This salt solution was removed by precipitating with 2 µl NaCO₂CH₃ and 60 µl 100% ethanol at -40 °C for 1 hour. The denatured DNA was collected by spinning at 12 000 g for 10 minutes and resuspended in 6 µl distilled water.

Sequencing was carried out using the T7 sequenase version 2.0 DNA polymerase kit from Amersham Life Science.

2.3.2 Annealing Reaction

The denatured DNA was pipetted into a 0.5 ml Eppendorf tube containing 2 µl sequenase reaction buffer (200 mM Tris-HCL, pH7.5, 100 mM MgCl₂, 250 mM NaCl) and 1 µl primer (Figure 2.1). The tube was heated for 2 minutes at 65 °C then slowly cooled to 35 °C over 15 minutes. In a microtitre plate 2 µl of dGTP, dATP, dTTP, dCTP termination mixes (80 µM of each dNTP, 8 µM of the required ddNTP and 50 mM NaCl) were pipetted into wells and incubated at 37 °C. Labelling mix was diluted 1:4 in distilled water and Sequenase polymerase was diluted 1:6 in dilution buffer (10 mM Tris-HCl, 5mM DTT, 0.5 mg/ ml BSA) to ensure the correct concentrations were present.

The radio labelled S³⁵ dATP (0.5 µl of 1500 Ci/mM S³⁵ dATP per reaction) was mixed with; 2 µL labelling mix (7.5 µM dGTP, 7.5 µM dCTP, 7.5 µM dTTP), 1 µl 0.1M DTT and 2 µl of sequenase polymerase. The labelling mix was then added to the cooled annealing reaction, mixed, and then 3.5 µl was added to each of the dNTPs termination mixes in the microtitre plate. This polymerisation reaction was stopped after 2 minutes with the addition of 4 µl stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol). The reactions in the microtitre plate were loaded onto a polyacrylamide gel or stored at -20 °C refrigeration. The microtitre plate was heated at 95 °C to denature the base pairing between the strands and 5 µl of each reaction was loaded onto a polyacrylamide gel.

2.3.3 Denaturing Polyacrylamide Gel Electrophoresis (PAGE)

Two glass sequencing plates were cleaned with alternating solutions of distilled water, acetone and methanol, to ensure that there were no impurities on the glass which would cause bubble formation when the gel was poured. The two plates were separated with 1 mm spacer bars and the edges of the plates were either taped together or held together with bulldog clips. The gel solution was prepared by dissolving 21 g urea (BDH), 0.05 g ammonium persulphate, 6 ml RapidGel-XL acrylamide gel, 5 ml 10X Sanger TNE buffer (Appendix 1), to a final volume of 50 ml in distilled water. Running hot water around the perimeter of the flask facilitated dissolution. The solution was left for 10 minutes to cool before 20 µl N,N,N',N'-tetramethylethylenediamine (TEMED) was added to initiate polymerisation.

The denatured DNA was loaded on to the polyacrylamide gel in the order G, A, T, C. Running buffer was loaded into the anode and cathode trays of the electrophoresis apparatus. PAGE was carried out for the required length of time depending on the length of the sequence. The two blue marker bands from the dyes in the stop solution gave a reference of how far the products had travelled. The two glass plates were slowly separated and the gel was then transferred to blotting paper, covered with cling film and then dried on a gel drier for 30 minutes. The cling film was removed from the dried gel, which was then taped into a gel cassette. Autoradiography film (Kodak Biomax MR) was inserted into the cassette, in the dark, and left for 24 hours before the film was removed, in the dark, and developed by a gel developer.

Approximately 200 bases were visible in each 4-lane sequence. They were read, taking care the gel was in the correct orientation, into Genebank, which checked that it was the required product. The sequence was entered into Simmonic software version 2000 and sequence manipulation, translation and phylogenetic analysis were carried out using the programme, Molecular Evolutionary Genetic Analysis version 1.02 and version 2.0 (Mega).

2.3.4 Use of dITP sequencing for Core sequences

Sequencing as above was carried out on the core sequences with the use of dITP instead of dGTP. In regions which are GC rich, such as the start of the core gene, secondary structure of the DNA forms, which is not fully denatured during electrophoresis. These fragments migrate faster causing bands that are

compressed together, which make the sequencing gel hard to decipher. Inosine is a base analogue of guanosine, which forms weaker secondary structures reducing problems with compression of bands on gels.

2.4 Use of restriction enzyme sites in primers to position DNA sequence in plasmid

Each of the internal primers used in the secondary PCR reactions contained restriction enzyme sites. This allowed the required DNA sequences of HCV to be cut out of the pTAg vector once the sequence was known and inserted in the correct orientation and frame into the expression vectors.

2.5 Expression of Recombinant Proteins

This work was carried out at Murex Biotech Ltd. by Mr S. Tucket, Ms R. Mason and Dr B. Rodgers. Sequences for the core, NS3 and NS5 regions from HCV genotypes 3 and 4 were subcloned into *E.coli* or baculovirus expression vectors as appropriate, using the restriction enzymes in the secondary primers. The NS3 and NS5 sequences of each genotype were ligated into *E.coli* vector pTrcHis (Invitrogen BV, Netherlands) and transformed into TOP10 cells. Cultures were grown in 2YT medium and expression of recombinant protein induced by the addition of IPTG to 1 mM. Cells were harvested 3 hours post induction and lysed by treatment with lysozyme and DNase in the presence of protease inhibitors.

The core sequence was transformed into the baculovirus expression vector pBlueBacHis2 (Invitrogen) and recombinant virus was derived by co-transfection into insect cells along with linear autographa californica nuclear polyhedrosis virus (AcNPV) DNA. Recombinant virus expressing core antigen, as judged by western blot, was plaque purified and amplified to give high titre virus stock. To express recombinant proteins, Sf. 9 insect cells were expanded in serum-free medium in an LH Series 210 bioreactor to approximately 2×10^8 cells/ ml and then infected with recombinant virus at a measure of infectivity (m.o.i.) of 2 units. After 48 hours, cells were harvested and nuclei isolated by lysis with 1% Nonidet P-40. The nuclear located recombinant protein was released by treatment with 0.2% sodium deoxycholate and digestion with DNase.

Both the *E.coli* and baculovirus expression vectors placed a tract of 6 histidine residues at the amino terminus of each recombinant protein. Recombinant proteins were purified by metal chelate chromatography on Probond nickel affinity resin (Invitrogen).

2.6 Preparation of antigen coated ELISA plates

To optimise plate coating, purified antigens were titrated to levels giving acceptable specificity and sensitivity and approximately equivalent amounts of protein/ well. Binding of antigen was assessed both by enzyme immunoassay with positive and negative sera and by probing for the histidine tag present on each protein using horseradish peroxidase chemically modified to bind nickel ions. All antigens were coated overnight in 50 mM Tris pH 8.5 containing 0.02% SDS. Plates were blocked by the addition of 2% degraded gelatin and dried.

The plates were tested using serum from blood donations from the Tooting Blood Transfusion Centre. The distribution of reactivity measured with these samples was similar for each antigen irrespective of genotype.

2.6.1 ELISA for detection of antibody to single antigens

The recombinant proteins of core, NS3 and NS5 from Murex Diagnostics production strain HCV-UK (of genotype 1b) and the equivalent proteins of core and NS3 of genotype 4a, and NS3 and NS5 of genotype 3a, were coated on to ELISA plates. Aliquots of 20 µl from each titration were added to 180 µl of sample diluent, mixed, then added to the ELISA well. A solution of 1:50 *E.coli* blocker was added to the diluent for all assays of NS3 genotype 2a and 3a antigens. This was necessary to prevent antibody, produced against *E.coli*, reacting with any *E.coli* proteins remaining after antigen purification. The plates were then incubated at 37 °C for one hour in a water bath. Following incubation the plates were washed five times with glycerate-borate wash buffer, before 100 µl of horseradish peroxidase labelled antibody to human IgG conjugate was added. After 30 minutes incubation in a water bath at 37 °C, the plates were washed five times with glycerate-borate wash buffer before the addition of 100 µl 3,3',5,5'-tetramethylbenzidine (TMB) substrate. Following a further 30 minutes incubation at 37°C in a water bath, 50 µl 2 M H₂SO₄ stop solution was added. The optical density (OD) of each plate was measured at 470 nm on a spectrophotometer. A 20 µl aliquot of anti-HCV negative plasma

used in the dilutions was used to blank the spectrophotometer to discount background serological reactivity. All assay plates included a high titre genotype 1a control.

2.6.2 ELISA for detection of antibody directed against genotype 3a Core, NS3, NS4 and NS5 antigens

The genotype 3 antigen plates were the nearest match to the Murex VK48 assay as could be provided. The genotype 3 plates contained recombinant protein from core region amino acids 1-140, NS3 region amino acids 1360-1454 and NS5 region amino acids 2238-2321 expressed as a tripartite fusion protein. Branched peptides from region 1 amino acids 1691-1708 and region 2 amino acids 1710-1728 (Bhattacharjee et al., 1995) of NS4 of genotype 4 were also coated onto the ELISA plates. The difference between the genotype 3 assay and the VK48 assay was that linear NS4 peptides were included rather than branched peptides.

The specificity and sensitivity of the plates was compared using samples from the local Tooting Blood Donor Centre. A coating level of approximately 200 ng/well of tripartite protein and 25 ng/well peptide gave a comparable sensitivity and specificity to the VK48 assay.

The test was performed as in 2.6.1 above.

2.6.3 Quantification of antibody levels

Antibody reactivity in each of the test sera to each recombinant protein was determined by titration and reference to a standard positive control as described (Dhaliwal et al., 1996). This method was based upon the observation that optical density over the range of values 0.02 to 1.0 shows a linear relationship to the concentration of antiserum. Therefore, antibody could be expressed relative to the reference control.

Calculation of levels of antibody, expressed here as units (u), could be represented by the following formula:

$$\text{Test antibody level} = \frac{\text{Test O.D.} \times \text{Reference concentration}}{\text{Reference O.D.} \times \text{Test concentration}}$$

For each antigen plate a number of control samples were titrated in serial dilutions in negative sera and 20 µl of each dilution tested in the assay. The optical density was measured for each dilution. A graph was plotted of antibody level against optical density measured (Figure 2.4).

Once each antigen had been tested to ensure that plates had even coating and a linear relationship of antibody level to optical density could be measured a relative antibody level for each sample for each antigen could be calculated.

To measure the antibody level in a sample relative to the standard for any given antigen, the control sample was titrated out in negative serum and duplicate aliquots of 20 µl added to each well in the upper two rows. The concentration of the titration depended of the sensitivity of the assay.

For each plate a graph was plotted of concentration of antibody to optical density measured figure 2.5.

Again each sample is titrated according to the sensitivity of the assay for single antigens titrations were 1:40 -16:40 for most samples, only those with O.D measured above 1.0 at the 1:640 dilution would need to be titrated further.

To measure the antibody level in a sample the OD in the linear range would be used for the calculation. If more than 1 titration gave an OD in the linear range the all would be calculated. If the difference in relative antibody level measured was greater than ten fold between the two dilutions then the sample would be repeated.

The antibody level relative to the control standard could be calculated using the titration concentration of the control, the measured OD at that concentration and the OD and concentration of the standard in the linear range of the graph. For example in figure 2.5 the control has an O.D of 1.1017 at 1:320 (0.003125) and 0.701 at 1:640 (0.0015625) dilution. The sample we wish to compare relative to the standard has an OD of 0.8 at a dilution of 1:640. Using the reference value of OD 0.701 at 1:640, these values are substituted into the formula above to give;

$$\text{Test antibody level} = \frac{\text{Test O.D.} \times \text{Reference concentration}}{\text{Reference O.D.} \times \text{Test concentration}}$$

$$\text{Test antibody level} = \frac{0.8. \times 0.0015625}{0.701 \times 0.0015625}$$

$$\text{Test antibody level} = 1.14$$

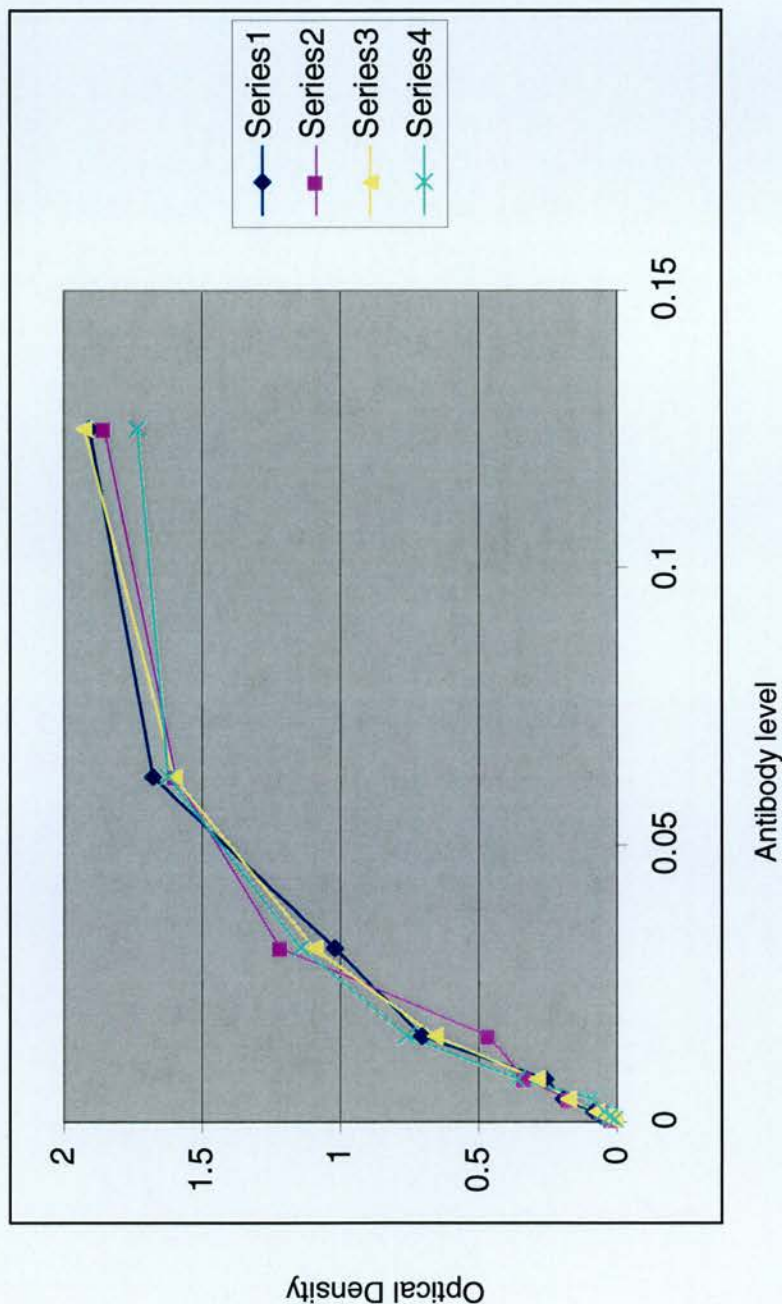


Figure 2.4 Correlation of Antibody in a sample against Optical Density measured.

One plate coated with antigen of each genotype and region was tested to ensure even coating and to check the relationship “concentration of antibody in a sample was linearly related to optical density measured”, until substrate saturation occurred. Four known high anti- HCV antibody titre samples were titrated 1:10- 1:2560 in HCV negative serum and 20 µl of each dilution tested. Each sample was tested in duplicate and 3 wells of negative control were used to set the spectrometer to discount background absorbency. The measured optical density was plotted against concentration. For all the plates measured optical density was linearly related to concentration of sample, between the measured OD values 0.2 -1.0 units. A linear relationship was found between OD measured and antibody level.

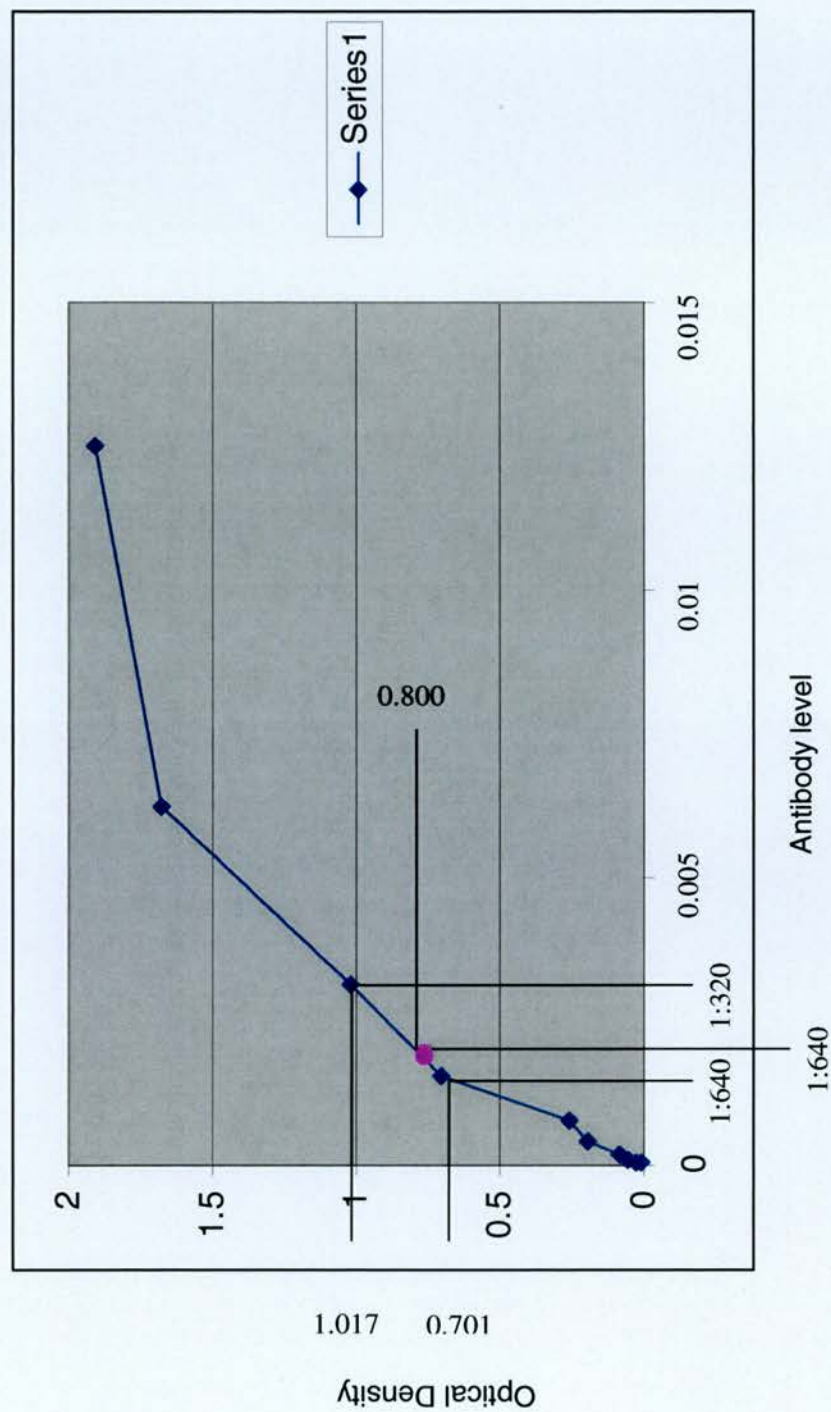


Figure 2.5 Concentration of antibody to optical density measured in the standard sample

2.6.4 Plate to plate variation

It was often found that the plates varied in intensity of colour change on different test occasions, by as much as 30%, despite the test conditions being identical. This phenomenon, despite the use of the control standard, caused values taken on one occasion to vary slightly on repeat testing.

Repeat testing of samples showed good correlations with the original antibody level result. A selective bias to any one genotype group, was avoided by ensuring each plate tested contained an equal number of samples of each genotype. This problem is unlikely to influence the results as the number of samples involved should balance out the anomaly.

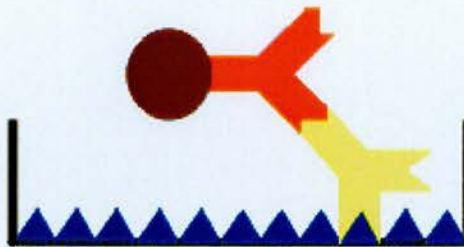
Plate is coated with antigen



Incubate human serum on antigen coated plate, IgG in the serum will bind to the antigen on the plate



Addition of conjugated anti-human IgG which binds to IgG which is bound to the antigen.



The conjugated enzyme indicates the presence of the bound compound by catalyses a colour change when a substrate is added

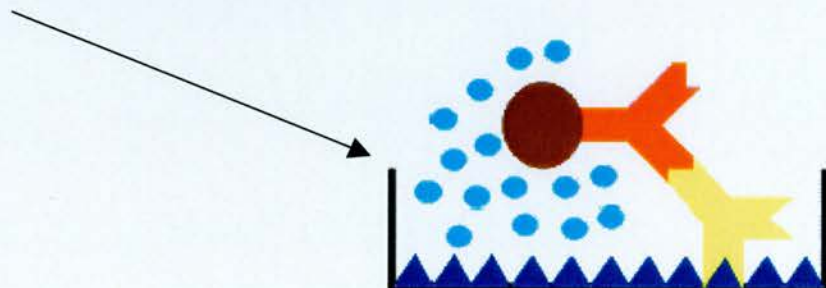
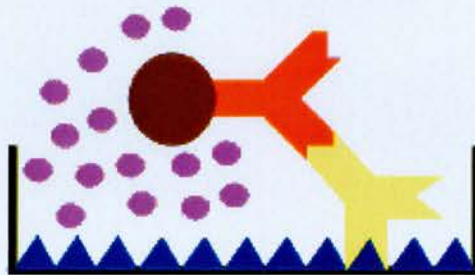


Figure 2.6 Construction of an Enzyme linked Immunosorbent Assay

2.7 Commercial ELISAs

2.7.1 Murex VK48

The Murex 3rd generation ELISA used contained recombinant proteins from core, NS3, NS4 and NS5 regions of HCV. The test procedure consisted of four stages: incubation of sample with antigen, addition of conjugate which binds to any anti-HCV antibodies from the sample, colour change of substrate catalysed by conjugated enzyme and reading of optical density.

Prior to carrying out the test procedure, all the reagents were allowed to reach room temperature and the solutions of conjugate, conjugate diluent, substrate and substrate diluent were mixed. In each test two volumes of the anti-HCV positive control and three volumes of the negative control were included.

The first stage of the test consisted of 20 µl of fresh or stored serum being mixed with 180 µl of the supplied diluent, and then dispensed into a well on the 96 well plate, which was covered and incubated at 37 °C in a water bath for one hour. During the incubation, antibodies contained in the sera bound to the antigens captured on the ELISA plate. The plate was washed four times with the supplied glycerate borate wash buffer, diluted 1:20 in distilled water, which removed any unbound serum antibodies. The second stage involved the addition of 100 µl conjugate (horseradish peroxidase labelled mouse monoclonal antibody to human immunoglobulin G protein) to each well that was covered and incubated under the above conditions for a further 30 minutes. The plate was washed four times to remove any unbound conjugate, before the addition of 100 µl substrate TMB and hydrogen peroxide. The reaction was allowed a 30 minute incubation before it was stopped by the addition of 50 µl of 2 M H₂SO₄ stop solution. A colour change of pink to blue occurred in the TMB in wells when anti-HCV antibody was present. The colour recorded was proportional to the amount of conjugate antibody bound and, therefore, proportional to the concentration of anti-HCV in the original serum sample. This colour change reaction was halted by the addition of sulphuric acid, changing the colour to yellow. The optical densities (OD) in each well were measured at 470 nm wavelength on a spectrophotometer. The cut-off OD was 0.6 units plus the mean OD of the three negative controls. Any OD above the cut-off was positive, anything below negative.

2.7.1.1 Changes made to the protocol above

The Murex VK48 protocol was modified when measuring the antibody level in a particular sample on this assay. The changes involved a run of 20 µl aliquots of a designated standard control in dilutions 1:320 - 1:160 000 in rows A1-10 and B1-10 and negative controls in A11, A12, B11 and B12. The sample tested was diluted 1:10 - 1:10 240 and 20 µl of these used in the wells. The protocol above was followed until reading the ODs in the wells. The spectrophotometer set at 470 nm was blanked, using one of the negative controls in wells A11, A12, B11 or B12, and then the absorbencies measured in each well. Then, using the formula shown in section 2.6.3 above, choosing a dilution within the range where the concentration and absorbency are linearly related, the relative antibody level in each sample was calculated.

2.7.2 Abbott 3rd Generation Enzyme Immunoassay (EIA)

The Hepatitis Reference Laboratory in Edinburgh used this commercial assay to test human sera for anti-HCV antibodies. A volume of 10 µl of test sample or control was pipetted into a test tube, 400 µl of specimen diluent was added and 200 µl of this mixture was then transferred to the reaction well of the assay plate. One recombinant HCV coated bead was added to each well, and then the plate was incubated for 1 hour at 40 °C under humid conditions. After incubation, the diluent was aspirated from around the beads and the beads were washed four times with deionised water. The beads were then incubated with 200 µl bovine and goat anti-human antibody conjugate at 40 °C for 30 minutes. The conjugate was removed by aspiration and the beads washed, before removing each bead to a labelled test tube. The substrate solution was mixed as directed: 1 o-phenylenediamine dihydrochloride (OPD) tablet in 5 ml substrate diluent, for every 13 beads used. To each sample test tube and the two substrate controls, 300 µl freshly prepared substrate was added. This was incubated for 30 minutes at room temperature before 1 ml of 1 M H₂SO₄ was added. The substrate control test tubes were used to blank and set the spectrometer at 490 nm. Three positive and three negative samples were tested per assay. The cut-off of the assay was determined as the mean of the three negative samples plus a quarter of the mean of the three positive samples.

2.7.3 Ortho 3rd generation EIA

The assay components were allowed to reach room temperature for 30 minutes prior to commencing this assay. The 1A well of the ELISA plate was the substrate blank. This meant that only substrate, and no other reagents, was added to this well. On each plate there was one substrate blank, two positive controls and three negative controls. A 200 µl volume of sample diluent was added to all wells except 1A, before 10 µl of sample or control was added into each well and mixed. The plate was incubated under humid conditions at 37 °C for 30 minutes, before washing five times with supplied buffer. The plate was blotted dry and 200 µl conjugate was added to all wells except 1A, and then incubated again at 37 °C for 30 minutes. Sufficient substrate OPD was freshly prepared. The ELISA plate was washed as before and 200 µl fresh substrate solution was added to all wells and incubated in the dark at room temperature for 30 minutes. The colour change was halted by the addition of 50 µl 2 M H₂SO₄ to all wells. A spectrometer was blanked using the substrate blank well 1A, before reading the absorbencies of all of the samples at 490 nm. The plate cut-off was determined as the mean of the 3 negatives plus 0.330 OD units. Any sample or control with an optical density measured greater than this value was considered positive.

2.7.3.1 Modifications to the Ortho 3rd generation EIA

The above assay was modified to allow relative antibody level measurements in a sample to be calculated. The standard control was titrated 1:10-1:160 000 and two aliquots of 20 µl volumes of each dilution were added to each plate for reference. Each sample tested was titrated 1:10-1:10 240 and 20 µl of each of these titrations was tested. The above protocol was followed until reading the ODs in the wells. The spectrometer, measuring wavelength 470 nm, was blanked using one of the negative control samples, and then the absorbencies were measured in each well. Then, using the formula described in section 2.6.3 above, choosing a dilution within the range where the concentration and absorbencies are linearly related, the relative antibody level in each sample was calculated. The serum samples were allowed to thaw, then were gently pipetted up and down to ensure homogeneity throughout the specimen. The standard control was titrated 1:320 - 1:10 240 in the anti-HCV antibody negative serum, two aliquots of 20 µl volumes of each dilution were added to each plate for

reference. Each sample tested was titrated 1:10 - 1:10 240 in negative serum; 20 µl of each of these titrations was tested. If the sample's measured optical density (OD) was less than 0.4 units, the sample was titrated 1:10, 1:20 and 1:40 and 20 µl of these dilutions was tested in the assay. Three aliquots of 20 µl of the anti-HCV negative serum in place of the negative control were included in each plate. The plate cut-off for each plate used was calculated as the mean of the 3 negatives plus 0.6 OD units. Anything above this value was considered positive. The antibody level in each sample was assigned relative to the standard sample as described previously in section 2.6.3. The antibody levels assigned to each sample were then divided by five. This represented a five fold loss of sensitivity.

The cut-off for each plate was calculated as an antibody level by the formula and by assigning the concentration of the control as one.

$$\text{Cut-off antibody level} = \frac{\text{Cut-off O.D.} \times \text{Reference concentration}}{\text{Reference O.D.} \times 1}$$

2.7.4 Chiron Recombinant Immunoblot Assay 3.0 (RIBA)

The RIBA assay was used as a confirmatory assay for samples already found to be positive by ELISA. This strip immunoblot was based on a western blot where immunogens were attached to a nitrocellulose strip and positive samples detected by enzyme catalysed colour change. The antigens used were recombinant proteins c33c (NS3 amino acids 1192-1457), NS5 (amino acids 2054-2995), synthetic peptides c100p (NS4 amino acids 1920-1935), 5-1-1p (NS4 amino acids 1694-1735) and c22p (core amino acids 10-53). Each antigenic region was coated as a separate band, allowing the specific antigen of anti-HCV reactivity to be observed. Both the c33c (expressed in *E.coli*) and NS5 (expressed in yeast *Saccharomyces cerevisiae*) antigens contain the tagged sequence of human superoxide dismutase hSOD. hSOD was also coated onto the strips to discount any reactivity with the expressed proteins that is really due to the reactivity to this sequence. Also there were two IgG control antigens blotted onto each RIBA strip.

A conjugated enzyme catalysed a colour change by the action of hydrogen peroxide and 4-chloro-1-naphthol creating a blue-black insoluble band on strip. The colour of the band was graded from 1 to 4 by comparison with a reference high and low titre standard.

The assay components were allowed to reach room temperature before beginning testing. The required number of strips were removed from sealed foil pouches and placed in the assay plate in their respective wells, one well per specimen and one well for the positive and negative kit controls required. A list of specimen numbers and corresponding RIBA strip numbers was prepared. RIBA specimen diluent (1 ml) was added to each sample well, then 20 µl of the appropriate specimen or control was pipetted to the corresponding well. The assay plate was placed on a plate rocker and rocked at 16-20 cycles per minute for 4 to 4.5 hours at room temperature. The specimen diluent was then aspirated and discarded, and was replaced with 1 ml of fresh specimen diluent and the plate restored to the rocker for a further 30 minutes. The specimen diluent was then removed and 1 ml of assay supplied working wash buffer was added to each well. The strips were removed from each well into wash vessels containing 30 ml of working wash buffer. The working wash buffer was removed and replaced a further 3 times, before the conjugate was added, 1 ml for each strip used. The wash vessel was rotated on a rotary shaker for 15 -20

minutes at room temperature. After incubation, the conjugate was decanted and the strips were washed again three times in 60 ml of working wash buffer. Working substrate 1 ml per strip was added to each wash vessel and the wash vessel was rotated on a rotary shaker for 15-20 minutes at room temperature. The substrate was then aspirated and the strips were washed twice in 60 ml distilled deionised water. The strips were removed from the wash vessel using forceps and transferred to absorbent paper to blot excess moisture. The strips were air dried in the dark for at least 30 minutes at room temperature. The results of the test were interpreted by comparing the intensity of any band present with the reference chart.

2.7.5 Murex Serotyping Assay

The assay utilised the finding that anti-HCV antibodies directed to the NS4 region are often type-specific. Each of the wells in the assay plate was coated with peptide from all six of the HCV genotypes. Solutions of the peptides in a ten fold higher concentration than that of the concentration of the assay wells were prepared. Of the solutions prepared, one contained all six of the genotypes' peptides. Another six solutions were prepared where each only contained five of the genotypes. The solutions were added to the wells so that in a row: one well had no competing peptide solution, one well had competing solution containing all genotypes, one well had competing solution from all genotypes except genotype 1, one well had competing solution from all genotypes except genotype 2 and so forth. When a sample was added to these wells, the type-specific antibody bound to the more concentrated peptides in solution rather than to the peptides coated on the solid phase of the assay. The type-specific antibody, which bound to antigen in solution, was washed away, with the exception of the well where the genotype, which corresponded to the particular genotype of the sample, was not contained in the competing solution. In that well the antibody bound more strongly to the solid phase antigen and was detected on incubation with conjugate and colour change of added substrate.

On commencing the assay all components were allowed to reach room temperature, the wash buffer was diluted 1:20 and the conjugate and substrate were mixed with their respective diluents. One strip of eight coated wells was required for each patient sample or control. Aliquots of 10 μ l of the appropriate competing solution (6 with one different genotype missing in each and one with

all genotypes included) were added to the relevant wells. Sample diluent (180 µl) was pipetted into each well, and then 10 µl of sample or control was added to each of the eight wells of one strip and mixed thoroughly. The wells were covered with a plate sealer and the plate incubated for 1 hour at 37 °C under humid conditions. After incubation, the plates were washed five times and 100 µl of conjugate added to each well. The plate was then returned to the incubator for a further 1 hour under humid conditions. The plates were again washed five times after the second incubation and 100 µl of substrate was added to each well. The plate was then incubated for 30 minutes at 37 °C under humid conditions. The plate was then removed and the colour development stopped by the addition of 50 µl of 2M H₂SO₄ to each well. The absorbencies of each well were measured at 450 nm using a microplate spectrometer. The genotype of the sample was determined from the well (or wells in a mixed infection) that gave a high absorbency value. If a high absorbency was measured in many or all wells, the test was repeated utilising only 5 µl of test serum.

2.8 Statistics

Statistics were analysed using the Systat version 5.0 statistical analysis computer package. Dot plot representations were used for all antibody level and ratio level comparison between genotypes, also median, mean, minimum and maximum values were calculated for each distribution. Kruskal-Wallis non-parametric analysis was used to determine the p values for comparisons between distributions. Correlations between different genotypes' antibody levels to core antigens were calculated using the non-parametric Spearman's rank correlation coefficient.

Chapter 3

3 Results 1

3.1 Determination of the Genotype-common and Genotype-specific Components of Antibody Response to Single Antigens

All current anti-HCV antibody screening assays are based on the most prevalence genotype sequence, type 1, despite the fact that six distinctly different genotypes exist. Countries where genotype 1 is not the most prevalent type report higher than expected occurrences of post transfusion hepatitis suggesting that current screening assays may not be sufficiently sensitive in detecting non type 1 infections. The extent of genotype-specific response to screening antigens is explored in this work to assess the impact of genotype on antibody detection by screening assay.

HCV sequence is highly variable, and the extent of the variability is constrained by the functional and structural aspects of each region. Four antigenic regions are used for commercial screening assays; core, NS3, NS4 and NS5. Regions such as the core are highly conserved between genotypes, but the NS regions are much more variable. This variability between the genotypes will be reflected in the antigenic regions and will affect the antibody response produced to these antigens and therefore influence detection on screening assays.

A screening assay utilising recombinant antigens of any other genotype has not been reported. Therefore the extent of reactivity in sera to homologous antigens, *ie.* genotype 4 antisera for genotype 4 antigens, has never been compared to sera reactivity for heterologous antigens, *ie.* genotype 4 sera for genotype 1 antigen.

Antibody reactivity is usually reported as the dilution of the last reactive sample in limiting dilution titration. However, this method is affected by antigen coating of wells and by the reaction conditions. Comparisons can only be made between samples on one plate and not relative to the antigen. Using limiting dilution, the antibody reactivity measured is on a logarithmic scale, therefore, the endpoints of very similar samples may vary by large amounts. To circumvent this problem a method was devised (Dhaliwal et al., 1996) which compares sample reactivity to a standard sample. The concentration of antibody in a sample is linearly related to the optical density measured in the substrate of the reaction well over the range 0.2 units to 1.0 until substrate saturation occurs.

This method allows reactivity in samples to be compared relative to each other and relative to each antigen.

Therefore, by comparing the reactivity to homologous and heterologous recombinant antigens, of each region, in infection with each genotype the type-specific and type-common component of response could be assessed.

3.2 Methods

3.2.1 Samples

Sera from HCV infected individuals were collected, RNA amplified by RT-PCR and the genotype determined by sequence analysis and by RFLP of the 5' non coding region (section 2.1.2.1). Samples were obtained from individuals infected with genotypes 3a (serum sample number LJ516) or 4a (serum sample numbers EG21, ED43) virus. cDNA sequences were amplified from the core, NS3, and NS5A regions of LJ516, the core region of EG21 and the NS3 and NS5A region of ED43. These sequences were cloned into transport vectors and sent to Murex Biotech. There, the sequences were subcloned into expression vectors and used for the synthesis of recombinant protein antigens (sections 2.4-2.6). Unfortunately, the expression vector core clone derived from LJ516 and the NS5A clone of ED43 did not express recombinant protein. The equivalent recombinant antigens from core (amino acids positions 1-140), NS3 (amino acids 1360-1454) and NS5 (amino acids 2238-2321) of HCV-UK (type 1b) were derived from an existing commercial anti-HCV assay (VK48).

Serum samples were obtained from 110 anti-HCV positive individuals with chronic hepatitis C attending hospital liver clinics in Edinburgh, London, Karachi and Cairo. Serum RNA was confirmed by RT-PCR and the genotypes were identified by RFLP of the 5' non coding region as 33 genotype 1b, 34 of genotype 3a and 43 of genotype 4a.

3.2.2 Antigenic Sequence

The generated clones from the amplified PCR products were given unique identification numbers. The clones which were used in the manufacture of the recombinant proteins were DX506, genotype 3a NS3; DX507, genotype 3a NS5; DX387, genotype 4a core and DX499 genotype 4a NS3. The sequences were entered into the GenBank sequence database. They were allocated the following GenBank unique identifying "accession" numbers DX 506, AF029297; DX507, AF029299; DX387, AF029298; DX499, AF029296. The

inferred sequence of the recombinant proteins expressed from the cloned PCR products was analysed and compared with other published sequences and grouped with the expected genotypes.

3.2.3 Assay techniques

ELISA plates coated with each of the recombinant proteins; genotype 1b core, NS3, NS5, genotype 3a NS3, NS5, genotype 4a core and NS3, were provided for estimation of antibody response with samples of each genotype. Murex Biotech Ltd also provided the reagents; sample diluent, 10 X wash buffer, conjugate and conjugate diluent, substrate and substrate diluent. ELISAs were carried out with each of the 110 patient serum samples, to estimate the relative antibody level in each sample. Two, one litre packs of anti-HCV negative serum were obtained from South West of Scotland Blood Transfusion Service for use as a diluent in titrations of the anti-HCV positive samples. A high antibody titre genotype 1a sample, obtained from a HCV infected patient attending the Edinburgh Royal Infirmary Liver Unit, was used as a positive control.

3.2.4 Quantification of antibody level

Antibody reactivity in each of the test sera to each recombinant protein was determined by titration and reference to a standard positive control (section 2.6.3).

3.2.5 Calculation of the Genotype-specific and Genotype-common Components of the Antigenic response.

The ratio of antibody levels measured to type-homologous and type-heterologous antigens indicated the relative proportions of type-specific and cross-reactive reactivity elicited on infection. Antigenicity for the purposes of this calculation represents the overall response to an antigen contained in an individual sample. This allows for any differences in levels of antibody produced and any differences in the antigen presentation when bound to the solid phase of an ELISA plate to be discounted.

A mathematical formula to estimate the proportion of type-specific and type-common components of response was derived by Dr Donald Smith (mathematical working listed in Appendix 3). The proportion of antibody reactivity that is cross-reactive between genotypes is measured by reciprocal assays of reactivity between sera and antigens from two genotypes, A and B. In the following relations, E_{BA} represents the antibody reactivity of a serum of genotype B with antigen of genotype A, A_{AB} is the overall antigenicity of the genotype A antigen relative to that of genotype B, and T_c and T_s are the proportions of the antibody response that are type-common and type-specific respectively, such that $T_c + T_s = 1$.

$$\frac{E_{BB}}{E_{BA}} = \frac{E_{BB}}{E_{BB} \times A_{AB} \times T_C} = \frac{1}{A_{AB} \times T_C}$$

$$\frac{E_{AB}}{E_{AA}} = \frac{E_{AA} \times (1/A_{AB}) \times T_C}{E_{AA}} = \frac{T_C}{A_{AB}}$$

Therefore:

$$\frac{E_{BB}/E_{BA}}{E_{AB}/E_{AA}} = \frac{A_{AB}}{A_{AB} \times (T_C)^2} = \frac{1}{(T_C)^2}$$

From this the proportion of type-common reactivity can be derived as follows:

$$T_C = \sqrt{\frac{E_{AB}/E_{AA}}{E_{BB}/E_{BA}}}$$

And

$$A_{AB} = \sqrt{\frac{1}{(E_{AA}/E_{AB}) \times (E_{BA}/E_{BB})}}$$

These relations make the assumption that the relative proportions of type-specific to type-common reactivity in a type A antiserum is the same as that found in a type B antiserum.

3.3 Results

3.3.1 Sequence analysis and phylogenetic comparison

Phylogenetic analysis of nucleotide sequences was used to confirm the identification of DX506 and DX507 as type 3a, and DX387 and DX499 as type 4a (Figure 3.2, Figure 3.4, Figure 3.6). The amino acid sequences of the recombinant proteins were deduced from the nucleotide sequences of the corresponding clones, (Figure 3.1, Figure 3.3, and Figure 3.5). Extensive amino acid differences existed in the non structural proteins while the structural core protein was more conserved. In the core protein 11 amino acids differed between genotype 1b and 4a over the 140 amino acid length (7.8% divergence). Substitutions were generally conservative and only one resulted in a change of ionic charge. The NS3 sequences exhibited more variability than the core protein. Genotype 1b differed from genotype 3a by 15 out of 95 amino acids (16% divergence) and from genotype 4a by 13 amino acids, while genotypes 3a and 4a differed from each other by 16 amino acids. NS5 was also more variable than core with 22 differences between genotypes 1b and 3a over a length of 83 amino acids (26% divergence). Seven of these substitutions affected the charge of the protein: three of basic amino acids for non-polar groups and two substitutions of acidic groups for uncharged groups, one acidic for non polar substitution and one unpolar to basic substitution.

Region	Genotype 1b Clone	Genotype 3a Clone	Genotype 4a Clone	Amino acid changes	Percentage divergence
Core	DX501	DX706		10	7.2
Core	DX501		DX387	11	7.9
Core		DX706	DX387	11	7.9
NS3	DX458	DX506		15	15.8
NS3	DX458		DX499	13	13.7
NS3		DX506	DX499	16	16.8
NS5	DXT1	DX507		22	26.5

Table 3.1 Divergence between genotype in the recombinant antigens

DX706 refers to a clone for genotype 3a core; this is discussed in chapter 4.

DX501	MSTNPKPQK	TKRNTNRRPQ	DVKFPGGGQI	VGGVYLLPRR	GPRLGVRATR
DX760I.....V....T..
DX387M.....
DX501	KTSESRQPRG	RRQPIPKARR	PEGRAWAQPG	YPWPPLYGNEG	MGWAGWLLSP
DX760E.....S.....	C.....
DX387S.....FA.....	C.....
DX501	RGSRPSTWGPT	DPRRRSRNLG	KVIDTLTCGF	GDLMGYIPLV	
DX760QN.....A..V.....		
DX387N.....L A.....A		

Figure 3.1 The inferred amino acid sequence of the recombinant core protein antigens (amino acids 1-140).

Sequence DX501 is the genotype 1b sequence in the Murex VK48 assay. Sequence DX387 is the inferred amino acid sequence after expressing the clone constructed from the genotype 4a viral sequence extracted from individual EG21. Sequence DX760 is the inferred amino acid sequence after expressing the clone constructed from the genotype 3a viral sequence extracted from individual C1085I discussed in chapter 4.

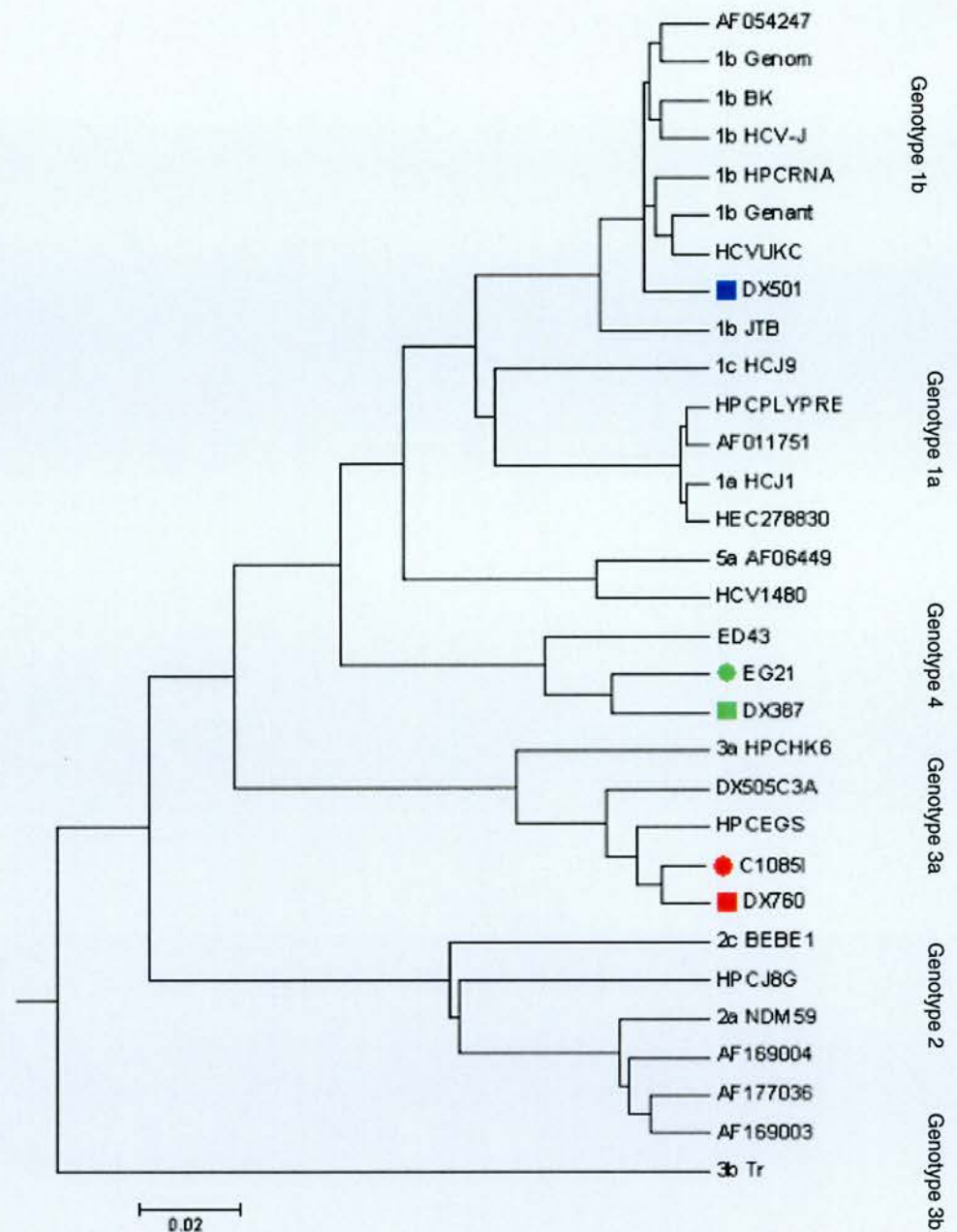


Figure 3.2 The phylogenetic relationship predicted using UPGMA analysis of the core region

Samples (C1085I and EG21) and the recombinant clones (DX760 and DX387) are compared with other published HCV sequences. The samples and the clones grouped with other published sequences of the expected genotype in all cases. The recombinant clone DX505C3A failed to express protein. The clone DX501 represents genotype 1 proteins.

DX458	PNIEEVAL	SNTGEIPFYG	KAIPETIKG	GRHLIFCHSK	KKCELAAKL
DX506	GSE.....ALL..I.S..
DX499	PT.....L.L...RQ.
DX458	VGLGINAVAY	YRGLDVSVIP	ASGDVVVVAT	DALMTGFTGD	FDSVIDC
DX506	R.M.L.....	TT.....L...
DX499	TS..L.....	T.....	Y.....Y

Figure 3.3 The inferred amino acid sequence of the recombinant NS3 proteins (amino acids 1360-1454).

Sequence DX458 is the genotype 1b sequence in the Murex VK48 assay. Sequence DX506 is the inferred amino acid sequence after expressing the clone constructed from the genotype 3a viral sequence extracted from individual LJ516. Sequence DX499 is the inferred amino acid sequence after expressing the clone constructed from the genotype 4a viral sequence extracted from individual ED43.

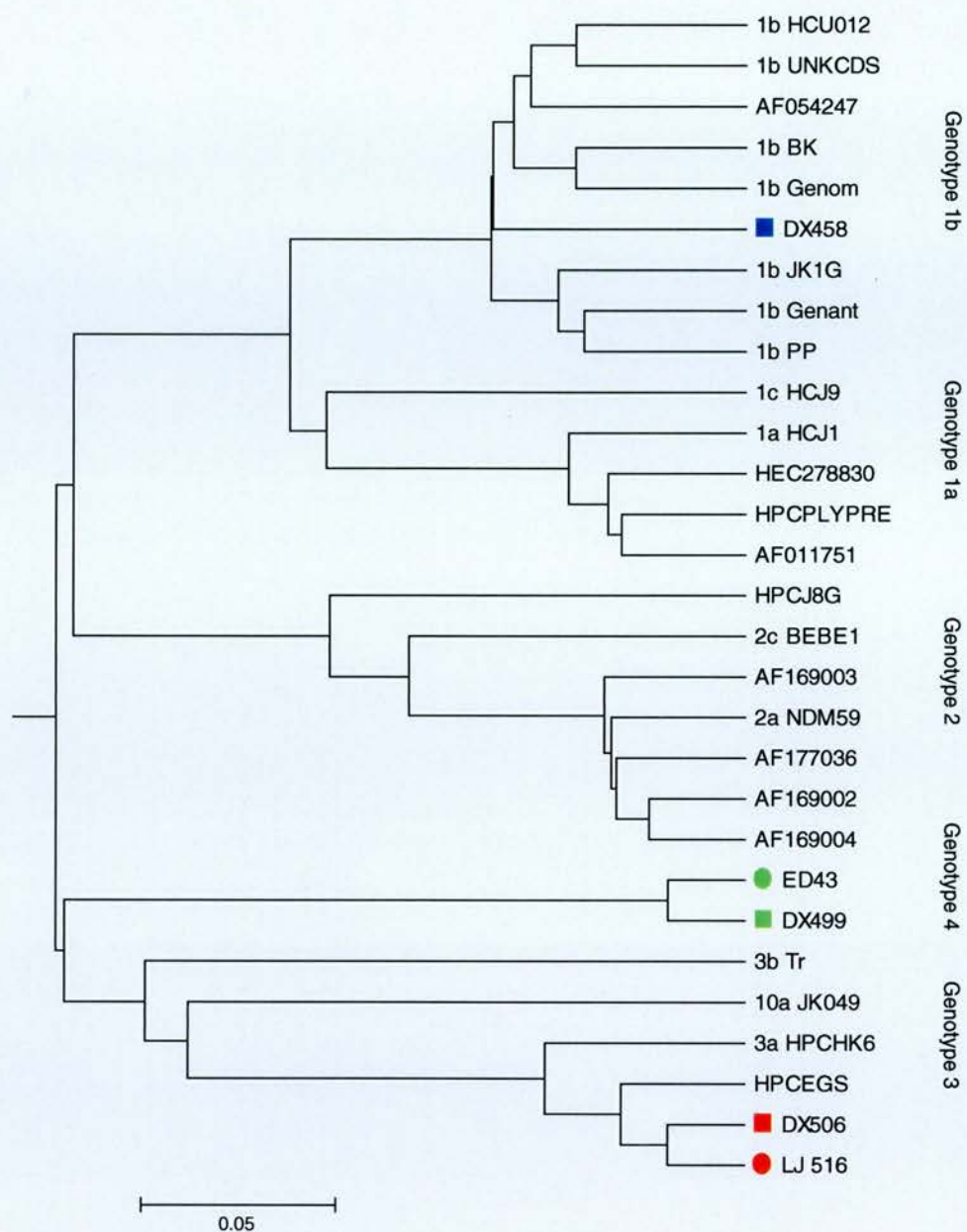


Figure 3.4 The phylogenetic relationship predicted using UPGMA analysis of the NS 3 region between the samples amplified and the recombinant clones.

Samples LJ516 and ED43 (sequence published as HCV 4APOLY) and the recombinant clones DX499 and DX506 compared to other published HCV sequences. Clone DX458 was used for the production of genotype 1 NS3 antigen.

DXT1	GGNITRVESE	NKVVVLD	SFD	PLRAE	EDER	EVS	VPAE	ILR	KSK	KFP	AAM	PIW	AR	PD	YNP	PLLE	SW	KD	PD	YVPP	VV	HG	CP	LP	PIK
DX507	.S.....	T.....	E	TT.DA	.L..	A..	C.K	.PP	.Y.P..DR..	A..	T....	A	...	QA							

Figure 3.5 The inferred amino acid sequence of the recombinant NS5 region protein antigens (amino acids 2238-2321)
Sequence DXT1 is the genotype 1b sequence in the Murex VK48 assay. Sequence DX507 is the inferred amino acid sequence after expressing the NS3 clone constructed from the genotype 3a viral sequence extracted from individual LJ516.

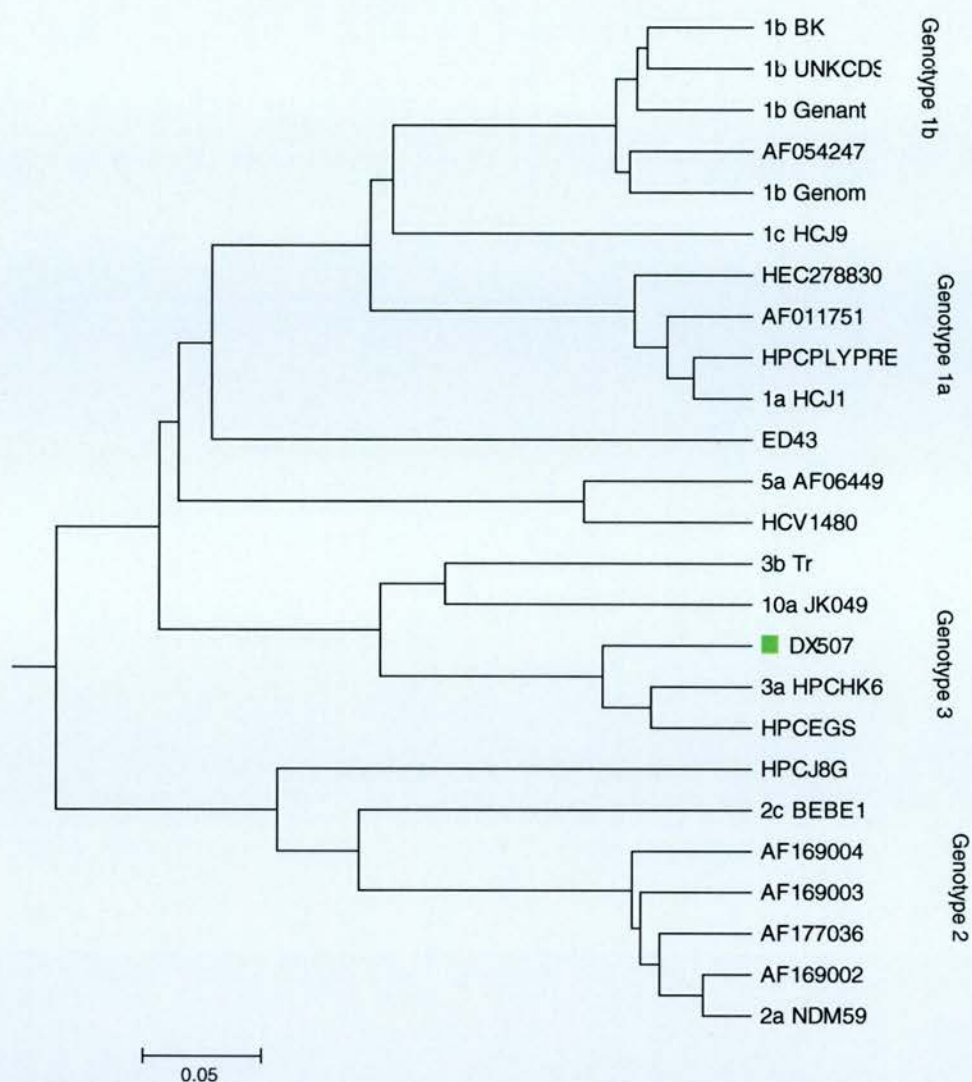


Figure 3.6 The phylogenetic relationship predicted using UPGMA analysis of the NS 5 region

The recombinant clones DX507 compared to other published HCV sequences. The genotype 4 NS3 sample sequence was not available nor was the sequence of the nucleotide genotype 1b clone.

3.3.2 Genotype dependence of serological reactivity.

The serological reactivity of the 110 collected sera from patients infected with HCV was measured. Sera collected from individuals infected with different genotypes: 33 genotype 1b infection samples, 34 genotype 3a infection samples and 43 genotype 4a infection samples, varied in their frequency of reactivity to core, NS3 and NS5 antigens of genotypes 1b 3a and 4a (Table 3.2).

In the core region, high frequencies of reactivity were observed for both type-homologous (95%-97%) and type-heterologous combinations (86%-97%), indicating the greater antigenicity of this region of the genome and/or a greater proportion of shared epitopes between genotypes.

For NS3, frequencies of reactivity for type 1b, 3a and 4a antisera to type-homologous NS3 proteins were 85%, 100% and 76% respectively, compared with a range of 27%-76% for type-heterologous combinations.

Similarly, the frequency of reactivity of genotype 1b sera with genotype 1b NS5 antigen (64%) was higher than that of heterologous sera (47% and 51%), as was reactivity to genotype 3a NS5 (58% type-homologous reactivity, compared with 24% and 45% for type-heterologous combinations).

3.3.3 Quantification of Antibody Levels

Each of the 110 sera samples was allocated an antibody level, relative to the control positive sample, for each antigen as described in section 2.6.3. The relative antibody level values were collated and analysed by Stystat statistical analysis package. For each antigen, the relative antibody levels for each genotype of samples were grouped and dot plots produced of genotype against relative antibody level. The median values for each distribution of relative antibody levels were calculated and the statistical significance between each of the distributions was calculated (Figure 3.7, Figure 3.8, Figure 3.9). There were significant differences in antibody reactivity to the type 1b core protein between genotype 1b samples and those of genotype 4a ($p=0.033$) but not of genotype 3a ($p=0.811$). The distribution of antibody levels directed to the genotype 4a core antigen showed no significant difference between genotypes, but the mean reactivity of genotype 4a samples was greater than that of heterologous genotypes.

ANTIGENS	Core		NS3		NS5	
	1b	4a	1b	3a	1b	3a
Antiserum						
Type 1b	<u>32/33</u>	<u>32/33</u>	<u>28/33</u>	<u>25/33</u>	<u>21/33</u>	<u>8/33</u>
	(97%)	(97%)	(85%)	(76%)	(64%)	(24%)
Type 3a	<u>33/34</u>	<u>30/34</u>	<u>15/34</u>	<u>33/33</u>	<u>16/34</u>	<u>18/31</u>
	(97%)	(88%)	(44%)	(100%)	(47%)	(58%)
Type 4a	<u>37/43</u>	<u>41/43</u>	<u>31/43</u>	<u>27/43</u>	<u>22/43</u>	<u>19/42</u>
	(86%)	(95%)	(72%)	(63%)	(51%)	(45%)

Table 3.2 Frequency of reactivity to core, NS3 and NS5 proteins.

Frequency values where genotype of sample and genotype of antigen are homologous are underlined

The reactivity directed to the NS3 antigen was much more genotype dependant. On the genotype 1b antigen plates, samples of genotype 1b had significantly higher reactivities than the genotype 3a ($p=0.004$) and 4a samples ($p=0.005$), also having a median value 100 times that of than of genotype 3a and 10 times greater than genotype 4a. The same trends were observed with the genotype 3a NS3 antigen where significant differences were observed between the distributions of the relative antibody level values of genotype 3a with that of 1b ($p=0.008$) and that of genotype 4a ($p<0.001$); the median value of genotype 3 samples was 4 times greater than that of genotype 1b and 20 times greater than genotype 4a. The distribution of relative antibody levels from samples from genotype 1b was not significantly different from the distribution of genotype 4a samples reactivity to genotype 4a NS3 antigen. However there was a significant difference in distribution between samples of genotype 3a and genotype 4a ($p<0.001$).

Only 54% of samples reacted with the NS5 genotype 1b antigen and 43% with the genotype 3a antigen. The median antibody level of genotype 1b sera (0.031) was eight fold higher than that of type 3a samples (0.004) and three fold higher for 1b antigen than type 4a (0.012). The distribution of antibody levels amongst genotype 1b samples was significantly higher than genotype 4a samples ($p=0.029$) but not the genotype 3a distribution.

Reactivity of type 3a sera against NS5 of genotype 3a was significantly greater than the type 1b or 4a sera ($p=0.002$ and $p=0.01$ respectively).

Antigen

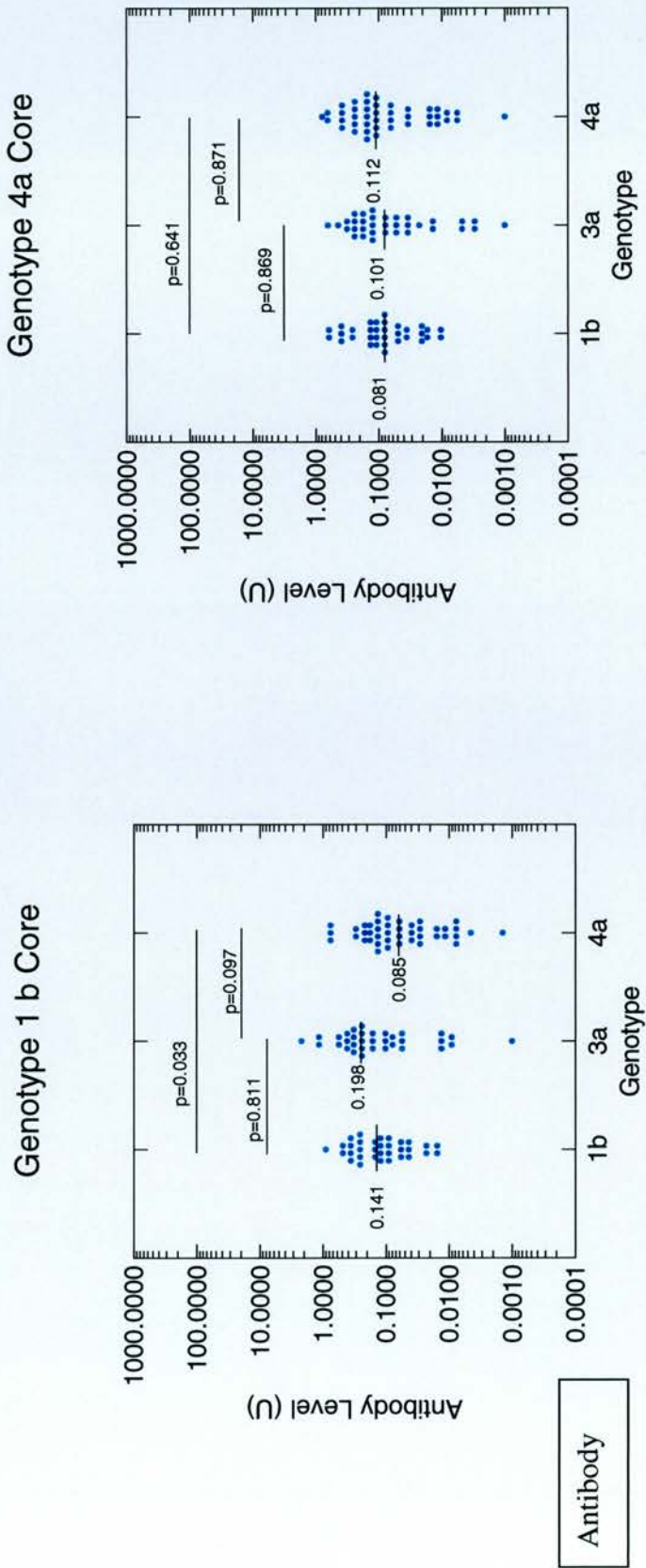


Figure 3.7 Distribution of antibody reactivity of sera from individuals infected with 1b, 3a and 4a virus with genotype 1b and 4a core antigen
Median values indicated by short horizontal bar. Pairwise comparison of distributions of values was carried out using the non-parametric Kruskal-Wallis test.

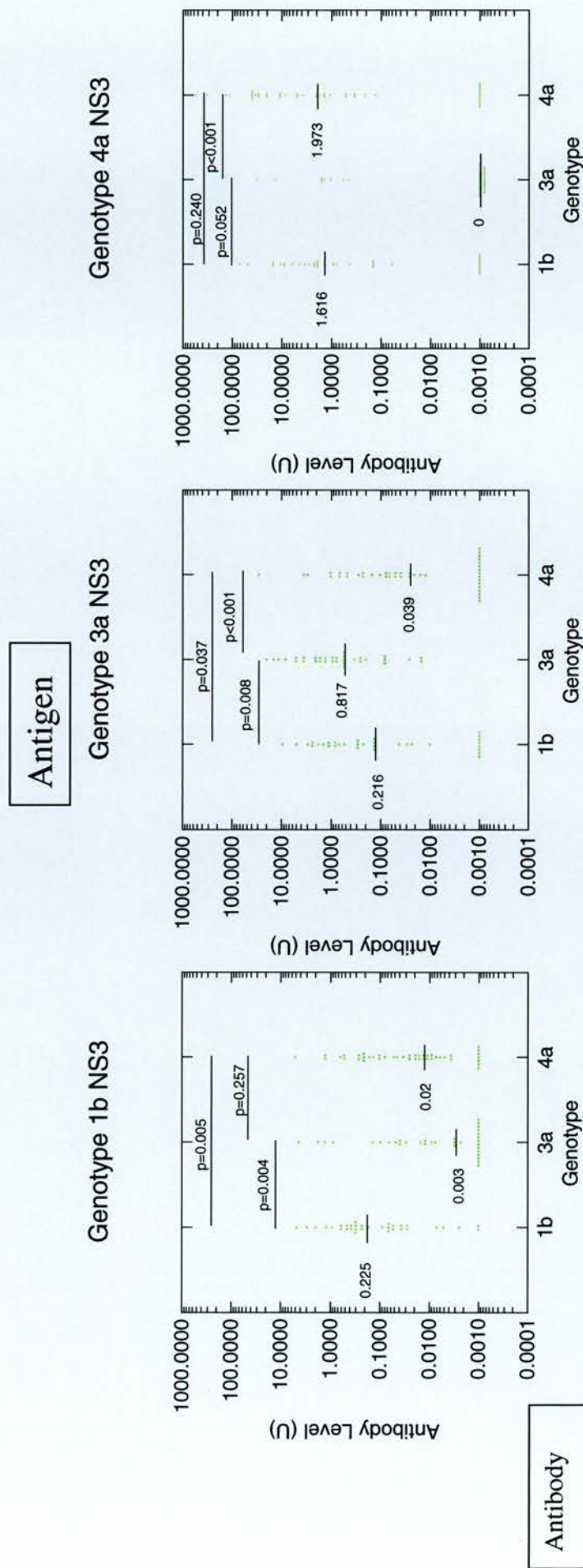


Figure 3.8 Distribution of antibody reactivity of sera from individuals infected with 1b, 3a and 4a virus with genotype 1b, 3a and 4a NS3 antigen
 Median values indicated by short horizontal bar. Pairwise comparison of distributions of values was carried out using the non-parametric Kruskal-Wallis test.

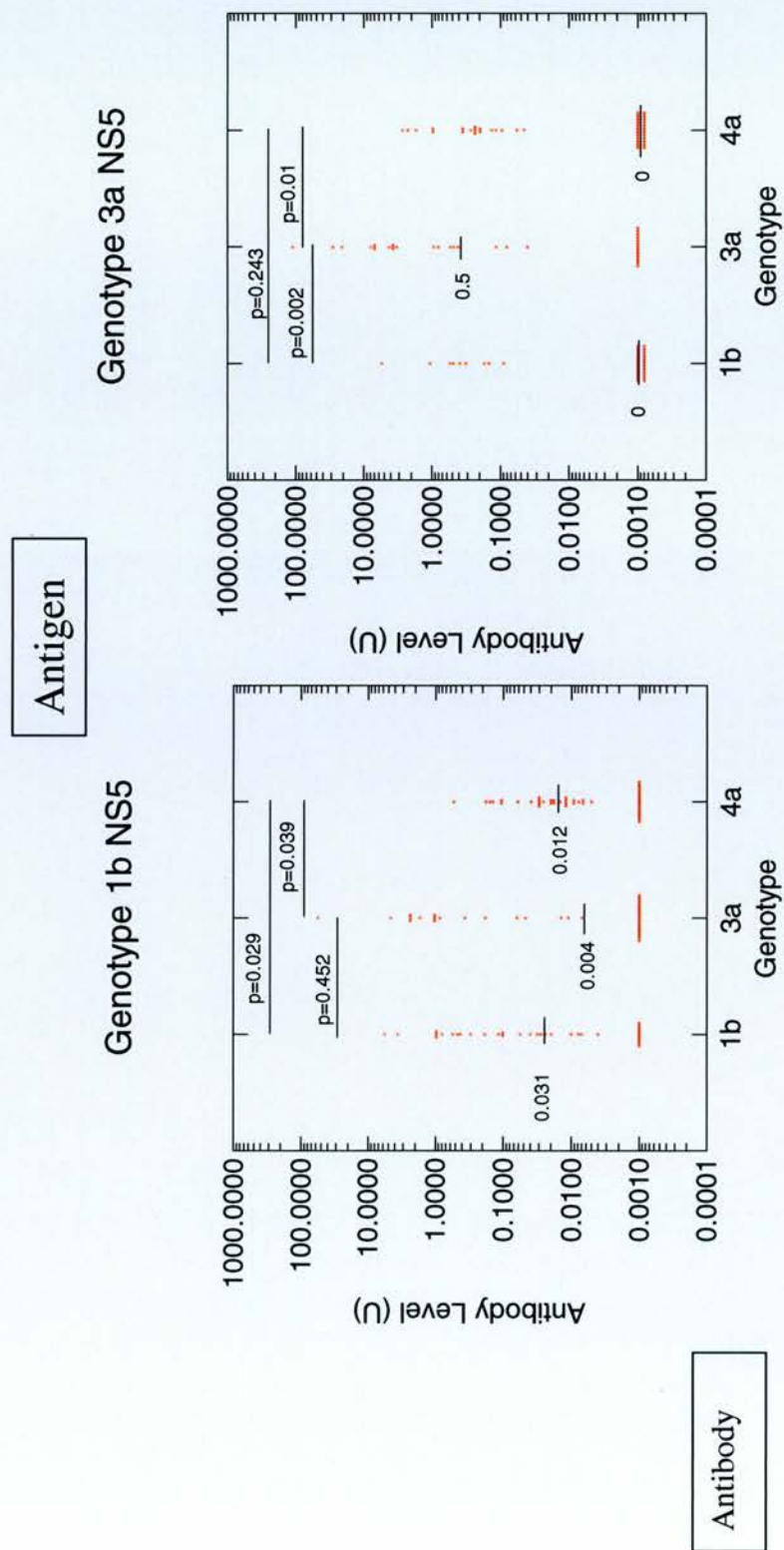


Figure 3.9 Distribution of antibody reactivity of sera from individuals infected with 1b, 3a and 4a virus with genotype 1b and 3a NS5 antigen

Median values indicated by short horizontal bar. Pairwise comparison of distributions of values was carried out using the non-parametric Kruskal-Wallis test.

3.3.4 Reproducibility of Results

To investigate the reproducibility of the method used to quantify antibody levels, each of the sera was assayed in replicate against the type 1b and 4a core proteins (Figure 3.10 and Figure 3.11). A close correlation was observed between the two measured antibody levels, with non-parametric correlation coefficients ranging from 0.877, 0.889 and 0.862 for type 1b, 3a and 4a antisera respectively. For the type 4a core antigens, the corresponding correlation coefficients were 0.784, 0.721 and 0.802

3.3.5 Correlation between Reactivity to Different Antigens

There was also a close correlation between reactivity to the core protein of type 1b with that to type 4a, with correlation coefficients of 0.834, 0.765 and 0.767 for type 1b, 3a and 4a antisera. Significant correlations were also consistently observed between the reactivity of antisera to NS3 proteins of types 1b, 3a and 4a, as well as between the NS5 proteins of type 1b and 3a. In contrast, there was little if any correlation between antibody reactivity to different regions of the genome. For example, the reactivity of sera from type 1b-infected individuals to the type 1b core protein showed no correlation with reactivity to type 1b NS3 or NS5 proteins (correlation coefficients of -0.118 and -0.181; Table 3.3). The only exceptions were weak correlations (0.443 and 0.471) between the reactivities to 3a NS3 and NS5 (but only for type 1b sera) and between NS5 of type 1b with NS3 of 4a (restricted to 3a sera).

Measurement of reproducibility of antibody levels to genotype 1 core antigen

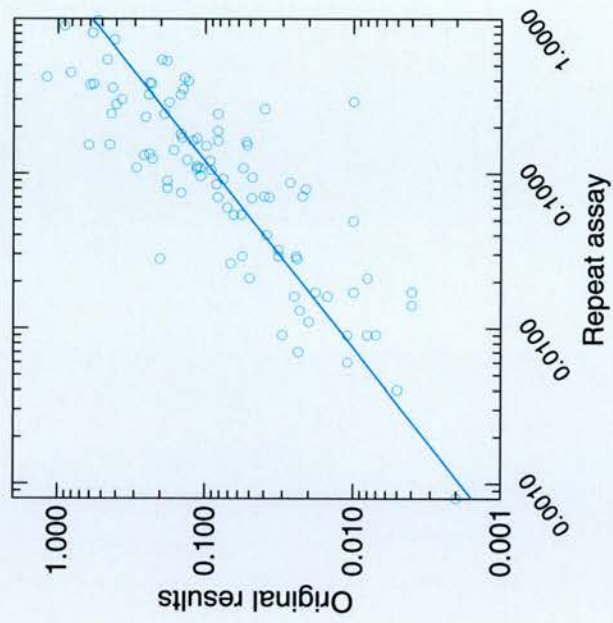


Figure 3.10 Measurement of the reproducibility of measurement of levels of antibody to type 1b core protein

Measurement of the reproducibility of antibody levels to genotype 4a core antigen

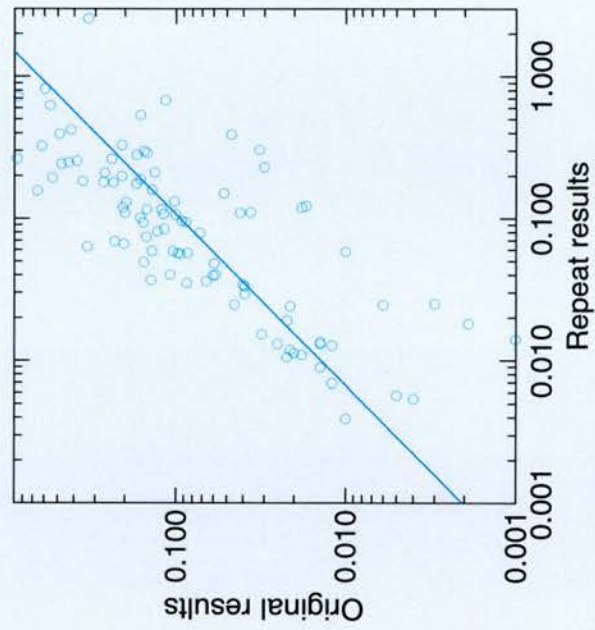


Figure 3.11 Measurement of the reproducibility of measurement of levels of antibody to type 4a core protein

Antigen and genotype	HCV genotype in serum	Correlation for the following antigens and genotypes ^a						
		Core		NS3		NS5		
		1b	4a	1b	3a	4a	1b	3a
Core								
1b	1b	(1)						
	3a	(1)						
	4a	(1)						
4a	1b	<u>0.834</u>	(1)					
	3a	<u>0.765</u>	(1)					
	4a	<u>0.767</u>	(1)					
NS3								
1b	1b	-0.118	0.023	(1)				
	3a	0.133	0.279	(1)				
	4a	0.100	-0.035	(1)				
3a	1b	-0.270	-0.066	<u>0.456</u>	(1)			
	3a	0.153	0.274	<u>0.363</u>	(1)			
	4a	0.159	0.030	<u>0.627</u>	(1)			
4a	1b	0.048	0.224	<u>0.710</u>	<u>0.518</u>	(1)		
	3a	0.215	0.130	<u>0.570</u>	<u>0.393</u>	(1)		
	4a	-0.058	-0.087	<u>0.497</u>	0.272	(1)		
NS5								
1b	1b	-0.181	-0.220	0.150	0.275	0.157	(1)	
	3a	0.066	0.000	0.218	0.265	<u>0.471</u>	(1)	
	4a	0.058	-0.192	0.069	0.168	0.055	(1)	
3a	1b	-0.141	-0.43	0.247	<u>0.443</u>	0.348	<u>0.630</u>	(1)
	3a	0.051	0.172	0.216	0.266	0.202	<u>0.649</u>	(1)
	4a	-0.076	-0.108	0.186	0.179	0.041	0.303	(1)

Table 3.3 Correlation between reactivity to different antigens.

^aSpearman's rank correlation coefficient

Separate values shown for antisera of each genotype

Significant values ($p < 0.05$) underlined.

3.3.6 Ratio of Reactivity to a Homologous Genotype antigen: Reactivity to a Heterologous Antigen.

The ability to measure the reactivity of the same antiserum to antigens of different genotypes allowed an estimation of the relative levels of genotype-specific and cross-reactive serological reactivity. For the core proteins, antibody levels of each of the type 1b and 4a sera to the genotype 1b antigen were divided by their levels to the genotype 4a antigen (Figure 3.12). The median ratio of reactivity to type 1b: type 4a antigens for the type 1b antisera was 1.49, compared with a median ratio of 0.809 for the type 4a sera. This indicates that type 1b sera react more strongly against the type 1b (homologous) core protein, while the type 4a sera reacted slightly more strongly against the type 4a antigen. If it were shown that both core proteins were coated equivalently on the solid phase, and showed equal antigenicity, then type-specific reactivity to these proteins could be calculated from the difference from the ratio of 1 expected from exclusively type-common reactivity. However, it is possible that some antigens are present in higher available concentrations than others, through differences in binding to the solid phase or solubility. These differences were taken into account using the derivation described. In this instance, the term E_{AB}/E_{AA} represents the median of the ratio of reactivity between type 4a sera with type 1b and type 4a antigens (0.809). The term E_{BB}/E_{BA} is similarly represented by the median ratio of the type 1b antisera (1.49). Therefore, the proportion of type-common reactivity can be calculated as 0.74, with type-specific reactivity forming the remainder of the reactivity (0.26).

The median ratio of reactivity of the type 3a sera to type 3a and 1b NS5 antigens (0.654) was substantially lower than that of type 1b sera (median of 4; table 3.4), therefore the proportions of type-common and type-specific reactivity were 40% and 60% respectively, ie. the majority of serological reactivity to this antigen was type-specific. Finally, three sets of pairwise comparisons can be made for the NS3 region, where antigens for all three genotypes were available. 62% of serological reactivity between type 1b and 3a proteins was type-specific, similar to 61% between type 1b and 4a, and 77% between type 3a and type 4a.

Ratio of Antibody Reactivity for Core Genotype 1b / Genotype 4a

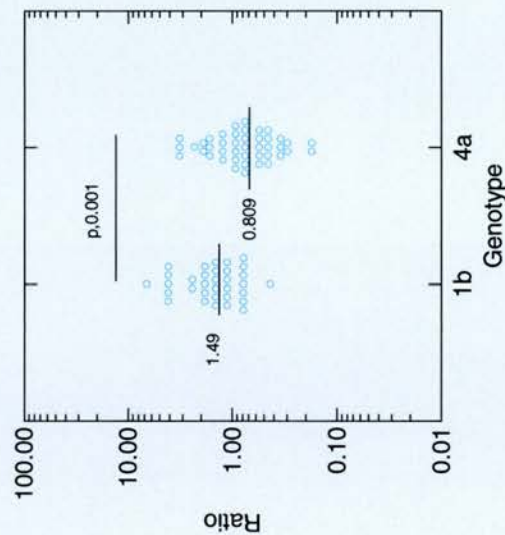


Figure 3.12 Ratios of reactivity of type 1b and 4a sera to type 1b /type 4a core antigens.

The values are the relative antibody level for each sample measured in the genotype 1b assay divided by the relative antibody level in the genotype 4a antigen assay.

Median ratios indicated by short horizontal bar. Pairwise comparison of distributions of values was carried out using the Kruskal-Wallis test. Ratios of reactivity to other antigens shown in table 3.4

Region	Antisera		Median ratios ^a		P value ^b	Percent		
	A	B	A	B		Type common reactivity (T _c)	Type specific reactivity (T _s)	Amino Acid Divergence ^c
Core	4a	1b	0.809	1.49	<0.001	74	26	7.2
NS5	3a	1b	0.654	4.0	0.014	40	60	26.5
NS3	3a	1b	0.053	0.373	0.001	38	62	15.8
	4a	1b	0.016	0.107	<0.001	39	61	13.7
	4a	3a	0.049	0.939	<0.001	23	77	16.8

Table 3.4 Type-specific and type-common serological reactivity to HCV core, NS 3 and NS5 Antigens

^aRatio of reactivity to antigens of genotypes A and B. The median relative antibody level of sample of genotype X in antigen A assay was divided by the median relative antibody level of samples of genotype X in antigen B assay. Where x is one of the genotypes.

^bComparison of the distribution of ratios of reactivity of individual antisera to antigens of genotypes A and B by the Kruskal –Wallis nonparametric test.

^cDegree of amino acid sequence divergence between antigens of genotypes A and B

3.4 Discussion on Single antigens

3.4.1 Antigenic variability of HCV

The aim of this study was to investigate the degree of type-specific serological reactivity to antigens used in current 3rd generation screening assays. The use of recombinant antigens expressed from different genotypes in the EIA allowed reciprocal measurements of type-homologous and type-heterologous reactivity, which provided a more rigorous assessment of the type-specific components of reactivity to core, NS3 and NS5. This addresses a potential criticism of previous investigations that showed weaker reactivity of sera from individuals infected with non-type 1 genotypes in either screening ELISA or to individual antigens in the RIBA confirmatory assay (Chan et al., 1991), (Claeys et al., 1995), (Dhaliwal et al., 1996), (Dow et al., 1996), (McOmish et al., 1993) and (Zein et al., 1995), in that there is a possibility that these observations resulted from a generally weaker serological response to infection than elicited by type 1. In the current study we were able to consistently show stronger reactivity of sera to antigens of homologous type than to heterologous types.

From pairwise reciprocal measurement of antibody reactivity (such as type 1b and 4a antisera against type 1b and 4a antigen), it was possible to quantify the relative contributions of type-specific and cross-reactive antibody reactivity using the relation derived in section 3.2.5. These calculations were independent of possible differences in the strength of the serological response elicited by infection with different genotypes, and were also independent of differences in antigen concentration or overall antigenicity between proteins of different genotypes (expressed as the ratio A_{AB}). This removes the potential criticism that the coating efficiency of antigens from different genotypes onto the solid phase was not compared prior to measurement of antibody levels. From this analysis we found a relationship between the degree of amino acid sequence divergence between recombinant proteins and their degree of cross-reactivity. The core protein was the most conserved and showed approximately 25% type-specific reactivity while the more divergent sequences NS3 and NS5 showed substantially greater proportions of type-specific reactivity. These results are consistent with previous comparisons of the type-specific component of reactivity to peptides corresponding to linear epitopes in NS4. By absorption in solution with peptides of heterologous genotypes, it was shown that reactivity

to type-homologous peptides was reduced, but rarely eliminated (Bhattacharjee et al., 1995; Simmonds et al., 1993b), and which therefore formed the basis of a sensitive and specific serological typing assay. The type-specific component of serological reactivity to NS4 and, in some studies to the core protein, have been also used in typing assays without cross-absorption, as it has been consistently observed that reactivity is stronger to type-homologous antigens in these (Dixit et al., 1995; Machida et al., 1992; Mondelli et al., 1994) and other regions (Zhang et al., 1995).

3.4.2 Implications for screening assays

The effect of the demonstrated antigenic variability of components of third generation screening assays on its overall sensitivity for screening is difficult to estimate with precision for two reasons. Individuals vary in their serological response to different antigens, and therefore in the extent to which this may cross-react with antigens of heterologous genotypes. For example, if reactivity were directed solely to the core protein, this would cross-react with antigens of heterologous genotypes, whereas sera that are monoreactive with NS3, such as found upon seroconversion, would be expected to be predominantly type-specific.

Furthermore, each of the three antigens investigated is likely to contain a range of linear and conformational epitopes, and these will vary in their degree of cross-reactivity. Recognition of different epitopes in the antigens may be one explanation of the wide range of ratios of reactivity to type-homologous and heterologous antigens observed between sera of the same genotype. In the extreme case, it is possible that reactivity confined to epitopes in the core protein that are type-specific would lead to poor or absent reactivity to the core protein of other genotypes. Conversely, the high degree of cross-reactivity observed between certain sera with NS3 or NS5 proteins of heterologous genotypes may have resulted from their recognition of shared epitopes.

The observed differences in reactivity of sera to homologous and heterologous proteins would only lead to false-negative results on serological screening if antibody levels in samples to be tested were close to the cut-off sensitivity of the assay. Low antibody levels, and reactivity to a restricted range of epitopes, such as those in NS3 are found in acutely infected individuals (Lelie et al., 1992), and it is likely that earlier detection of seroconversions, and therefore a reduction in the "window period" associated with non-type 1 infection, may be achieved using assays containing NS3 and other antigens from a wider range of genotypes.

- **A genotype specific response is directed at each antigen used in current screening assays.**

Chapter 4

4 Results 2

4.1 Determining the Genotype-Common and Genotype-Specific Components of Antibody Response to Combined Antigens.

Having found that an element of type-specific and cross-reactive immunity to each HCV antigen exists in each individual's serum, the logical progression was to measure the type-specific and type-common component to the combination of all HCV antigens in a screening assay. A genotype 3 based screening ELISA was constructed, to compare serum reactivity with the genotype 1 commercial assay, VK48. Serum samples were obtained from individuals infected with genotype 1, 2, 3 and 4 infections and the antibody level in each sample was measured, as before, (see section 2.6.3), relative to the standard control for each screening assay. The extent of the type-specific and type-common humoral immunity to these HCV antigens was determined.

4.2 Methods

4.2.1 Samples

The sera studied were obtained from blood transfusion centres and had been sent to the Molecular Virology Department at Edinburgh University for supplemental testing. These sera came from individuals identified, during routine donation screening, as HCV infected, during routine donation screening or from follow-up of recipients of contaminated blood or blood products. All of the samples were confirmed as containing viral RNA. The genotype in each sample was identified by RFLP of the 5' NCR as described in section 2.1.2.1. The subtypes of genotype 1 were further distinguished by RFLP using BstU1 (see section 2.1.2.2). The samples consisted of 36 genotype 1 (of these 10 were subtype 1a and 26 were subtype 1b), 7 genotype 2, 23 genotype 3 and 10 genotype 4. Samples of genotype 1 and genotype 3 infections were obtained from the Republic of Ireland Blood Transfusion Services from individuals whose main risk factor for infection was receiving contaminated anti-Rhesus D immunoglobulin. These two cohorts were particularly well matched for sex, age, health and HCV disease progression.

4.2.2 Antigenic Sequence

In addition to sample LJ516, sample C1085I was used in the production of genotype 3a recombinant protein antigen. Baculovirus expression vector clone DX505C3A of the LJ516 core sequence did not produce recombinant antigen. Other clones were created in the hope of expressing genotype 3 core protein. Sample C1085I belonged to a patient identified as having been the recipient of a batch of contaminated anti-Rhesus D immunoglobulin. Sequence analysis of the viral genome identified the virus genotype as type 3a, this was confirmed when this isolate grouped with other genotype 3 samples in phylogenetic analysis (Figure 3.2). The expression vector clone DX760, containing the amplified core sequence of C1085I alone, also did not express sufficient protein. The recombinant protein in the experimental genotype 3 screening assay was expressed as a tripartite fusion protein of core, NS3 and NS5. The protein consisted of sequences from the clones DX760, DX506 and DX507, ie, core region amino acids 1-140 from individual C1085I and NS3 region amino acids 1360-1454, NS5A regions amino acids 2234-2321 from individual LJ516. The predicted amino acid sequence of these clones is shown in figure 3.1, figure 3.3 and figure 3.5. The sequence of the genotype 1b antigens in the VK48 screening assay are also recorded in these tables as DX501, DX458 and DXT1. Branched synthetic peptides from regions NS4A and NS4B amino acids 1691-1708 and 1710-1728 of both genotype 1 and 3 were also incorporated in the assay figure 4.1.

TYPE	REGION 1	REGION 2
Type 1	KPAIIPDREV LYREFDEM	ECSQHLPYIE QGMMLAEQF
Type 1	...V.....A.....
Type 1	R..VV.....	
Type 1	R..V.....	
Type 2	RAV.A..K.. ..EA....	..ASKAAL.. E.QRM..ML
Type 2	RVVVT..K.I ..EA....	..ASRAAL.. E.QRI..ML
Type 2	RTV.A..K.. ..EA....	..ASRTAL.. E.HRR..ML
Type 2	RAVVA..K.. ..EA....	
Type 3	...LV..K.. ..QQY...AA.....AQVI.H..
Type 4	Q..V..... ..QQ.....	...K...LV. H.LQ.....
Type 5	R..... ..QQ.....	...TS...MD EAPAI.G..
Type 6	...VV.....I ..QQ.....	...R.I..LA E.QQI....

Figure 4.1 The amino acid sequence of the NS4 branched peptides (region 1 amino acids 16910 1708 and region 2 1710-1729).

4.2.3 Assay

The 92 serum samples were removed from -40°C storage and allowed to defrost. The thawed samples were titrated in negative serum in dilutions 1/10 - 1/10 240. Negative serum was obtained from the Scottish National Blood Transfusion Service and the same serum was used throughout this experiment. The standard control, a high antibody titre positive sample that was used in the single antigen plate analysis, was again used in this study. This reference standard control was titrated in the same negative serum; dilutions 1/320 - 1/81920 were added to each ELISA plate. Two wells of negative serum were present on each plate as a negative control. These were also used as the negative blank to discount for background reactivity. The ELISA plates were supplied by Murex Biotech: the commercially available genotype 1b based VK48 ELISA and the genotype 3 antigen coated plate BHC129. The genotype 3 ELISA was as near a match to the VK48 assay in terms of specificity and sensitivity as could be provided. However, the genotype 1b plate contained linear NS4 peptides whereas the genotype 3 plate contained branched peptides. The assays were carried out with Murex-supplied reagents (as described in section 2.6.2). The optical density observed in the wells was read at 470 nm.

4.2.4 Measurement of Antibody Level

The reactivity of samples to the commercially available Murex Biotech HCV screening assay (VK48) was compared with the experimental genotype 3a antigen assay, designated reference code BHC129. The OD measured in the reference control titrations was plotted against level of dilution to ensure a linear relationship between OD measured and concentration of antibody present. Each sample was assigned an antibody level relative to the standard control sample as previously described for single antigens (section 2.6.3). The samples were tested on the plates with a mixture of genotypes on each plate to prevent plate-to-plate variation from influencing the results.

4.2.5 Ratio of Reactivity

The extent of type-specific and cross-reactive reactivity could be estimated by comparing the reactivity of antisera to type-homologous and type-heterologous antigens, assuming the antigens were equivalent. The reactivity in each sample was converted into an antibody level by comparison with the standard control sample as described in section 2.6.3. The distribution of the relative antibody levels in samples of each genotype was analysed. The median of the distributions of the genotype 1 and type 3 samples were used in the formula derived in section 3.2.5 to estimate the extent of type-specific and type-common reactivity to antigens in a screening assay.

4.3 Results

4.3.1 Sequence

The amino acid sequence of the recombinant protein of genotype 3a core was deduced from the nucleotide sequence of the constituent clone DX760. The sequence is shown in figure 3.1. Pair wise analysis produced the figure, which confirmed the sequence groups together with other sequences known to be genotype 3 (Figure 3.2).

Within region 1 of the NS4 peptides (table 4.1), there was a high degree of divergence between genotype 1b sequence and genotype 3 sequence. The sequence of NS3 and NS5 are the same as discussed with the single antigens in section 3.3.1.

Region	Genotype 1b Clone	Genotype 3a Clone	Amino acid changes	Percentage divergence
Core	DX501	DX760	10	7.2
NS3	DX458	DX506	15	15.8
NS4 Region 1			6	33
NS4 Region 2			7	37
NS5	DXT1	DX507	22	26.5

Table 4.1 Divergence between genotype in the recombinant antigens

4.3.2 Genotype dependence of serological reactivity and quantitation of antibody levels

4.3.2.1 Genotype 1b antigens (VK48)

The distributions of antibody levels for sera with each genotype were plotted (Figure 4.2). The median value for type 1a was 0.268, subtype b 0.185, for type 2: 1.604, for type 3: 0.151 and for type 4: 0.809. Significant differences were observed between genotype 1 and 2, 1 and 3, 2 and 3.

4.3.2.2 Genotype 3a antigens (BHC129)

Relative antibody levels were plotted against sera with genotype for type 3 antigens (Figure 4.3).). The median value for type 1 subtype a was 0.084, b 0.19, for type 2: 3.04, for type 3: 0.708 and for type 4: 0.809. Significant differences were observed between genotype 1 and 3 and between genotypes 2 and 3.

4.3.3 Correlation between reactivity to different antigen

The correlation coefficient was found to be 0.86 between the relative antibody level of a sample in the VK48 assay against the level measured in the BHC129 assay, which was high considering the study by Dhaliwal et al., 1996 had found a correlation coefficient of 0.787 between the type 1b assay and the Ortho type 1a assay (Figure 4.4)

Antibody Reactivity on the VK 48 Genotype 1b Assay

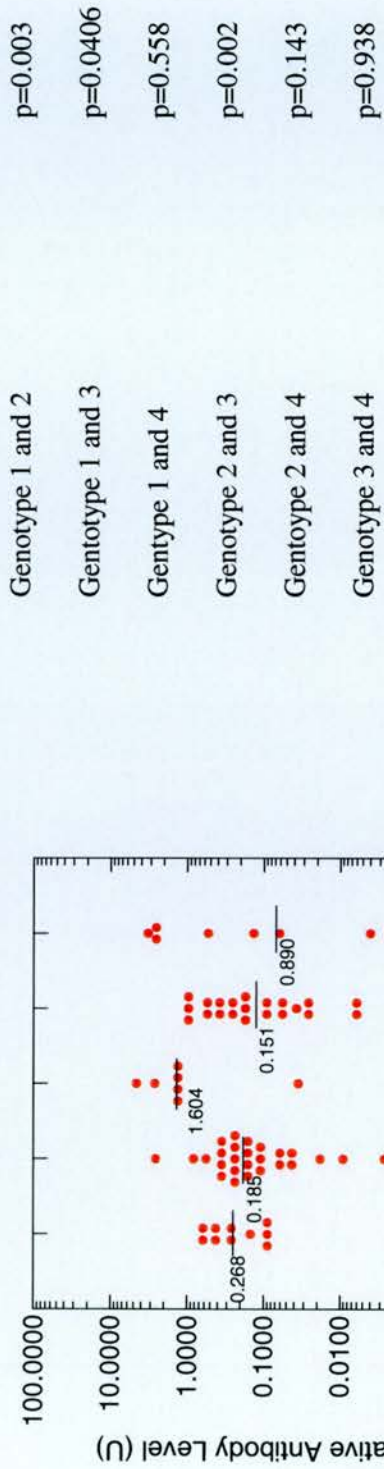


Figure 4.2 Distribution of antibody reactivity from samples of genotype 1 (n=36), genotype 2 (n=7), genotype 3 (n=23) and genotype 4 (n=10) to Antigens of the genotype 1 VK48 Screening Assay.

Antibody Reactivity on the BHC129 Genotype 3a Assay

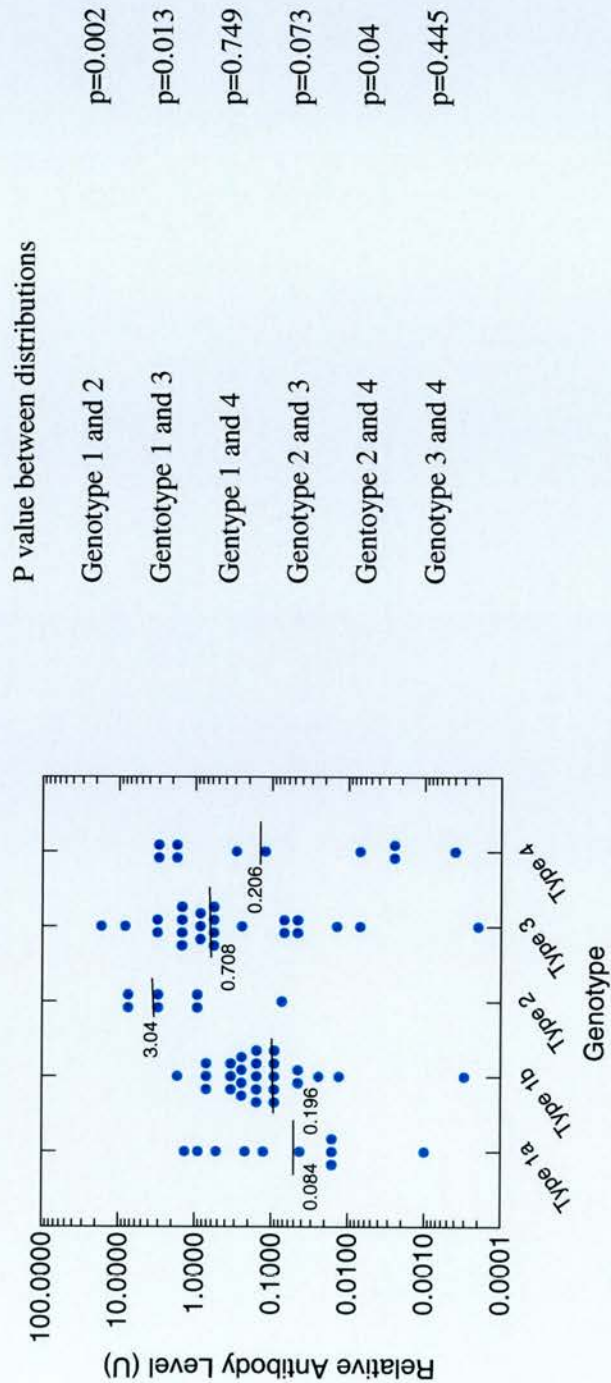


Figure 4.3 Distribution of antibody reactivity from samples of genotype 1 (n=36), genotype 2 (n=7), genotype 3 (n=23) and genotype 4 (n=10) to Antigens of the genotype 3 BHC129 Recombinant Assay.

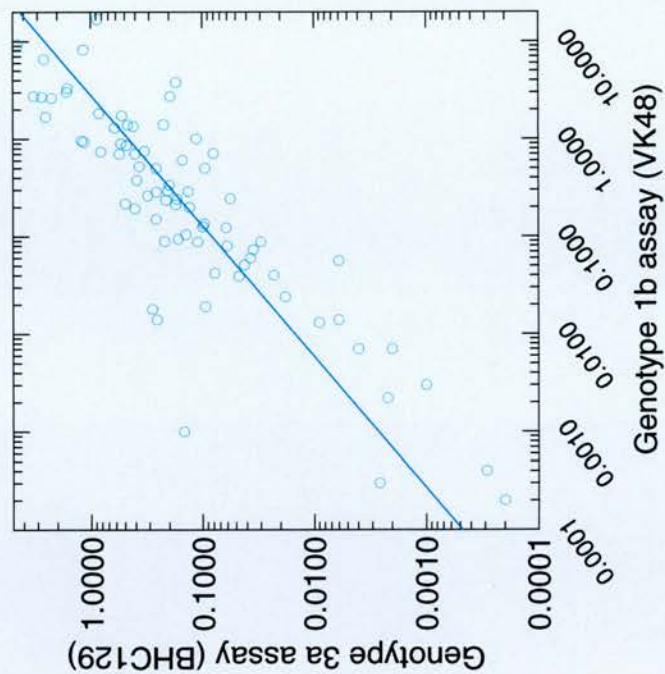


Figure 4.4 The Correlation between antibody reactivity to genotype 3a antigens and reactivity to genotype 1b antigens.

The correlation coefficient was 0.86 which was high considering the previous Dhaliwal study had found a correlation coefficient of 0.787 between the type 1b assay and the Ortho type 1a assay.

4.3.4 Ratio of Reactivity to a Homologous Genotype antigen: Reactivity to a Heterologous Antigen.

Using the methodology described in section 3.2.5 (Figure 4.5), the median values of both genotypes 1 (combined 1a and 1b) and genotype 3 were substituted in the formula;

$$T_C = \sqrt{\frac{E_{AB}/E_{AA}}{E_{BB}/E_{BA}}}$$

$$T_C = \sqrt{\frac{0.286}{0.966}}$$

The type-common component was calculated as 54% and the type-specific as 46%.

4.4 Discussion on combined antigens

This confirms the results observed with each antigen that a substantial portion of an antibody response is directed at type-specific epitopes.

- **A genotype specific response is directed at the combination of antigen used in current screening assays.**

Ratio of Reactivity in Serum to Genotype 1b Antigens and Genotype 3a Antigens

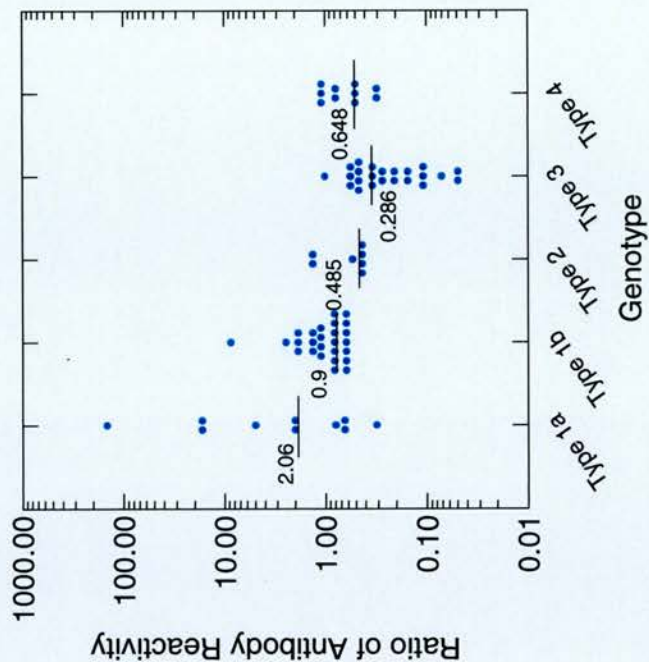


Figure 4.5 Ratio of Reactivity to type 1b antigens compared to type 3a antigens.

The ratio values are on a log10 scale on the y axis and the genotypes on the x axis. The median ratio values are shown by a bar. A significant difference was observed between distributions of genotype 1 and genotype 3 ($p<0.001$)

Chapter 5

5 Results 3

5.1 Introduction

Having found a proportion of the HCV antibody response is directed at type-specific epitopes, I set out to investigate the effect this had on detection with commercial assays. Simulating the 5-fold loss in sensitivity observed in non-genotype 1 infection in commercial assays, I investigated the theoretical effect on detection. I used the recombinant antigens to screen for individuals who were seronegative with commercial assay but whose infection could be confirmed by molecular biology. Using the recombinant antigens of the predominant infecting genotypes, I screened blood donors and patients with cryptogenic hepatitis in Pakistan and blood donations from immigrant Egyptians in Saudi Arabia. Having found no evidence of “missed” infections I investigated the possibility that recombinant antigens could be used to aid confirmation of indeterminant results. The samples available for analysis are listed in table 5.1.

Section	Number	Origin of samples	Comments
5.2	101	Dublin and Cork Blood Transfusion service	91 PCR positive, 20 PCR negative Samples from patients identified as receiving anti-RhD immunoglobulin contaminated with HCV genotype 1b
5.3	55	Cairo Liver Centre	50 Egyptian blood donors known negative for anti-HCV antibody by second generation Ortho EIA 5 antibody positive individuals
5.3	723	Brentwood Blood Donor centre	3 rd Generation Ortho EIA Anti-HCV screen negative
5.3	1547	Department of Pathology Riyadh Armed Forces Hospital	2 nd generation Ortho EIA Anti-HCV screen negative 693 from Saudi Arabian Nationals, 134 from Sudanese Nationals, 99 from Yemenis Nationals 621 from Egyptian Nationals
5.4	891	Karachi Blood Transfusion Service	887 no markers, 3 anti-HBV antibody positive, 5 anti-HCV antibody positive in anti-HCV antibody 2 nd generation assay, 3 borderline in anti-HCV antibody 2 nd generation assay, 3 elevated ALT
5.5	249	Scottish Blood Transfusion Service	155 Anti-HCV antibody positive indeterminate samples 94 control anti-HCV, HBV, HIV antibody negative
5.6	160	Aga Khan University Hospital Liver Unit, Karachi	41 cryptogenic hepatitis 25 fulminant hepatitis negative for anti-HCV (in 2 nd generation assay) and HBV antibody 17 anti-HBV antibody positive but anti-HCV antibody negative 69 anti-HBV antibody negative but anti-HCV antibody positive 8 anti-HCV and HBV antibody positive
5.7	20	Malmö University, Malmö, Sweden	10 patients genotype 3a HCV infection 2 or more sera available 10 control including 4 HCV PCR positive but anti-HCV serologically negative
5.8	3	Scottish Blood Transfusion Service	Anti-HCV antibody positive, PCR negative
5.8	3	German Blood Transfusion Service, Hagen Germany	Anti-HCV antibody positive, PCR negative
5.8	1	Rheinische Friedrich-Wilhelms University in Bonn	Serum was PCR positive for HCV but anti-HCV negative.

Table 5.1 Description of the source and number of sera samples available for study with the single antigen and combined genotype 3a screening ELISA

5.2 Theoretical under-detection of anti-HCV antibody-positive samples of other genotypes.

5.2.1 Introduction

Previous studies by Dhaliwal and colleagues (Dhaliwal et al., 1996) compared serological responses from individuals infected with viruses of genotypes 1, 2 and 3. The results showed the difference between genotype 1 and genotype 2 were samples statistically significant with both the Ortho 3rd generation EIA (adjusted difference = 4.12, $p=0.005$), and the Murex VK48 assay (adjusted difference = 3.72, $p=0.014$). These results include an adjustment to account for the 10.2-fold difference in reactivity found between PCR positive and PCR negative samples. Additionally, the responses of genotype 1 and genotype 3 sera were significantly different. The reactivity in the Ortho 3rd generation assay differed by an adjusted difference of 4.48 ($p<0.00001$) and in the Murex VK48 by 2.35 ($p=0.007$). This meant the detected antibody responses, measured in antibody units relative to a standard sample, in individuals infected with genotypes 1 and 3 were between two- and five-fold lower than the response of individuals infected with genotype 1 virus.

The effect of this 2 to 5 fold loss in sensitivity and the effect on the detection rate in commercial assays were investigated by previous colleagues (unpublished results). Their experiment was devised whereby samples from individuals infected with genotype 1b were diluted 1:4.5 in the specimen diluent and the reactivity measured in an Ortho 3rd generation test. This experiment mimicked the 4.48- fold difference in relative antibody levels observed between genotype 1 and genotype 3 samples.

The population that was studied were all infected from a single source over a limited time frame. In this cohort, after the 17 years since infection, the broad spectrum of outcomes of infection was represented. The samples were from individuals who had received an injection of anti-Rhesus D immunoglobulin, between April 1977 and October 1978, which had been manufactured from a plasma pool, which contained a HCV positive donation. The infected individual who had made the donation, which had contaminated the blood product, was traced. An archived specimen of the original sample was tested by polymerase chain reaction (PCR) and was found to contain viral sequence. The subtype of the virus was found to be 1b, by sequence analysis of NCR, core and NS5.

The extent of resolved infection and chronic infection was representative of the infection pattern observed in the general population. For this reason PCR positive and PCR negative samples were studied. By observing samples from genotype 1 infected individuals, diluted to represent the level of reactivity observed in genotype 3 samples, the sensitivity of the commercial assay with non genotype 1 samples could be estimated.

Contemporary samples of 101 recipients of this blood product were tested. The individuals tested had already been identified by the BTS as being 3rd generation Ortho EIA positive and recombinant immunoassay (RIBA) assay confirmed as positive for anti-HCV antibody. PCR results were available and RNA sequencing of several regions of the genome had been carried out on the PCR positive samples. These individuals had completed a medical history questionnaire and no other risk factors for HCV infection had been identified. The samples were tested in 3rd generation Ortho EIA according to the standard operating procedures, with the exception that the sample was diluted 1:90 in sample diluent instead of the protocol specified 1:20 dilution. At this 1:4.5 dilution, 16 of the 101 samples were below the positive cut-off value for this screening assay and 3 samples were close to this value. These 19 samples were PCR negative for viral RNA.

This experiment used dilution in sample diluent rather than dilution in negative serum. The effects of this on the results were unknown, but since the assay was designed to have a 20 µl volume of serum in each well and to allow comparison with the 20 µl serum in both the control positive and negative samples.

To overcome these uncertainties with the previous colleagues experimental design. I repeated this work. This time, instead of measuring the absorbance at a certain dilution, the antibody level in each sample was calculated relative to a standard control sample. By this method the antibody level in each sample could be examined and the effect of a 5-fold loss in sensitivity on the results could be established.

5.2.2 Samples

<u>Origin of samples</u>	<u>Number</u>	<u>Comments</u>
Dublin and Cork Blood Transfusion Service	111	91 PCR positive 20 PCR negative Samples from patients identified as receiving anti-RhD immunoglobulin contaminated with HCV genotype 1b

The serum samples for this study were from the Dublin and Cork BTS, from the cohort of individuals who had been exposed to HCV contaminated anti-Rhesus D immunoglobulin in 1977 or 1978. The 3rd generation EIA result, RIBA score and PCR status of each of the samples was available. The NS5 region of each of the PCR positive samples had been sequenced. All of the sequences were representative of 1b subtype. Those who were PCR negative, but seropositive, had documented evidence of receiving the contaminated anti- Rhesus-D and can, in the absence of other risk factors, been assumed also to have been infected with genotype 1b. The contemporary blood samples from these women were taken as described in section 2.1.1.1, and then shipped on dry ice to the Molecular Virology Laboratory for analysis.

One hundred and eleven samples were analysed in this study. This consisted of 91 PCR positive samples and 20 PCR negative samples, and contained 46 of the samples (31 PCR positive, 15 PCR negative samples) from the earlier experiment.

5.2.3 Assays

<u>Assays</u>	<u>Interpretation of results</u>
Ortho EIA	Plate cut-off = O.D. of the mean of 3 negative samples plus 0.6

The 3rd generation Ortho EIA was carried out according to the manufacturer's instructions (section 2.7.3), but modified to allow the calculation of the relative antibody level in each sample as described (section 2.7.3.1).

The Ortho 3rd generation assay was obtained through the SNBTS who use this assay for routine screening of all blood donations. The standard sample used to assign an antibody level to each test sample was a large volume high titre antibody positive serum sample. The anti-HCV antibody negative serum was

obtained from the SNBTS. The same negative donation was used for all dilutions in this experiment. The Ortho kit positive control was used according to the manufacturer's instructions. The anti-HCV antibody negative serum was used in place of the kit negative control and was used to calculate the plate cut-off values.

5.2.4 Methods

The Ortho Assay was performed as described in section 2.7.3.1. The antibody level in each sample was assigned relative to the standard sample as described previously in section 2.6.3. The antibody levels assigned to each sample were then divided by five. This represented a five fold loss of sensitivity.

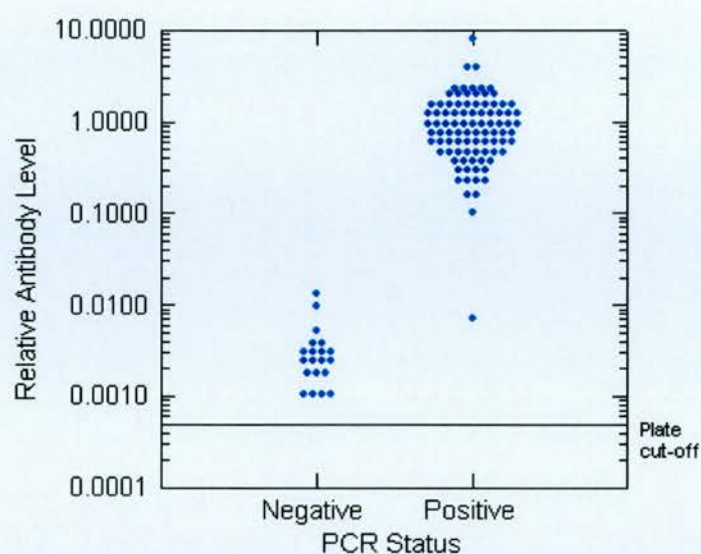
A dot plot was generated to illustrate the antibody levels in the samples and the relative antibody level, which represented the positive cut-off level, was indicated with a line.

5.2.5 Results

All 111 samples were assigned antibody levels, which were greater than the calculated plate cut-off antibody level value. However, when the antibody level was divided by five, 14 samples had antibody levels which were below the positive cut-off value, expressed as a relative antibody level. The distribution of antibody levels in the PCR positive samples was significantly different to those of PCR negative samples ($p < 0.001$).

The results are shown in figure 5.1 and figure 5.2. The 14 samples which became negative at a five-fold reduction were all samples which were PCR negative. The minimum relative antibody level measure was 0.000987 and the maximum was 7.920 relative antibody units. At a 1/5 decrease the minimum relative antibody level measure was 0.000197 and the maximum was 1.584 relative antibody units. The mean antibody level in PCR positive samples was 1.121 compared to 0.003321 in PCR negative samples. At a 1/5 dilution the mean antibody level in PCR positive samples was 0.224 compared to 0.00664 in PCR negative samples. The lowest relative antibody level recorded in a PCR positive sample was 0.007 units. Therefore, in this experiment for a PCR positive sample to fall below the cut-off limit, it would require a 13.2 fold loss in antibody level.

Samples tested on Ortho EIA test at normal dilution

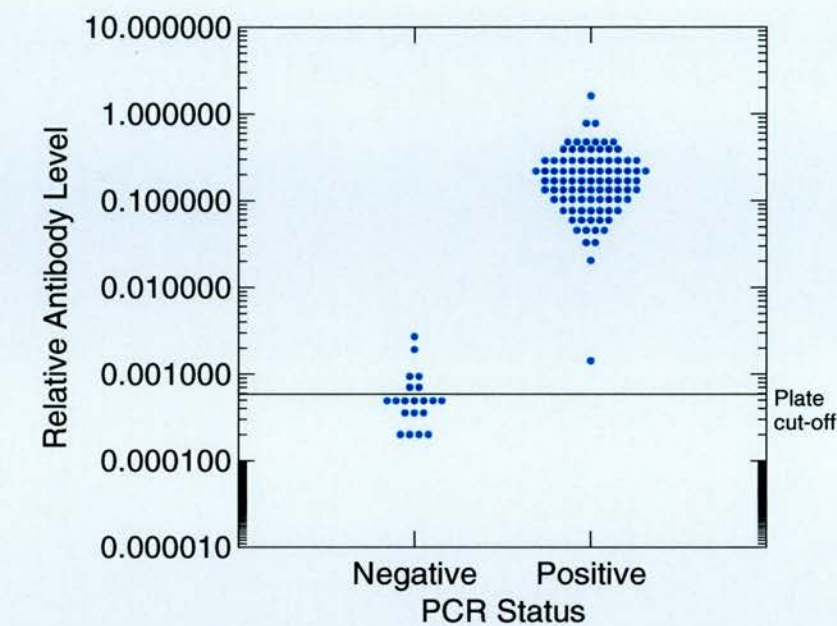


	All Samples	PCR Positive	PCR Negative
Minimum	0.000987	0.007	0.000987
Maximum	7.920	7.920	0.013
Mean	0.919	1.121	0.003321
Median	0.709	0.892	0.00253

Figure 5.1 Antibody levels in 111 HCV genotype 1b infected anti-Rhesus D recipients.

The antibody level of each sample measured is represented by one dot. The PCR status of the sample on the x-axis is plotted against the antibody level on the y-axis, which is a log₁₀ scale. The average cut-off level for the assay expressed as an antibody level is represented by the line drawn at 0.00053 relative antibody units. All 111 samples have antibody levels above the assay cut-off.

Samples tested on Othro EIA test at 1:5 dilution



	All Samples	PCR Positive	PCR Negative
Minimum	0.000197	0.001	0.000197
Maximum	1.584	1.584	0.0026
Mean	0.184	0.224	0.00664
Median	0.142	0.178	0.00506

Figure 5.2 Antibody levels in 111 HCV genotype 1b infected anti-Rhesus D recipients divided by five to represent a five-fold reduction in assay sensitivity. The antibody level of each sample measured is represented by one dot. The PCR status of the sample on the x-axis is plotted against the antibody level on the y-axis, which is a log₁₀ scale. The average cut-off level for the assay expressed as an antibody level is represented by the line drawn at 0.00053 relative antibody units. Fourteen of the 111 samples have antibody levels below the assay cut-off. These samples are PCR negative.

5.2.6 Interpretation

From these results with samples from individuals infected with genotype 1, there is evidence that the current screening EIA tests may not detect a number of PCR negative samples of other genotypes. The samples here are of PCR negative individuals, perhaps recovering from infection, where the antibody response is declining. Although PCR negative, the samples that were missed were RIBA confirmed. The referral guidelines at the time of this work suggested individuals who have an EIA positive result, which is confirmed by RIBA testing, should be referred to a Hepatologist or Gastroenterologist. This indicated that individuals infected with other genotypes who required further follow-up would not receive this care because their serum samples would be missed in the current assays. Another consequence of not detecting these samples in a blood transfusion setting would be that the previous donations would not be checked for infectivity and the recipients of such donations would not, therefore, be recalled.

The PCR positive sample with the lowest relative antibody level was 13.2 times higher than the antibody level plate cut-off value. This suggests that PCR positive donations of other genotypes would be detected despite the five-fold loss of sensitivity in current assays. However, the majority of the individuals in this study were relatively healthy and not co-infected with HBV or HIV. Perhaps immune suppression as a result of co-infection, other disease manifestations or immunosuppressive drug therapy may combine to produce an antibody level below the cut-off.

- **A five fold reduction in the sensitivity of anti-HCV antibody detection by Ortho 3rd generation assay results in under-detection of individuals with resolved HCV infection.**
- **A 13.5 loss in sensitivity of this assay would result in under-detection of active HCV infection.**

5.3 Screening donor populations in Cairo, Egypt and Riyadh, Saudi Arabia

5.3.1 Introduction

If screening with commercial assays was currently missing samples from individuals infected with HCV of genotypes other than genotype 1, then this would account for the cases of post transfusion hepatitis still being reported in regions where HCV genotype 1 is not the predominant genotype of infection. Egypt has a very high prevalence of HCV infection due to the lack of adequately sterilised medical equipment during a WHO Schistosomiasis eradication campaign. This large reservoir of HCV carriers results in a continual low level of HCV transmissions (Nafeh et al., 2003). The predominant genotype of infection is genotype 4. Therefore, this was the ideal population to study, although, unfortunately, access to a large number of blood donor samples from Egypt could not be arranged (only 50 samples were analysed). The closest alternative was to use blood donations from individuals in Saudi Arabia where there are a number of immigrant Egyptians (1547 samples were analysed). These samples were screened for genotype-specific reactivity, using genotype 4 antigens, which corresponded to the genotype 1 antigens in commercial assays. Personnel from Murex Biotech Ltd carried out the majority of this work. However, analysis of the data and PCR testing of samples from the Egyptian and Saudi Arabian blood donors were carried out in the laboratory in Edinburgh.

5.3.2 Samples

<u>Origin of samples</u>	<u>Number</u>	<u>Comments</u>
Cairo Liver Centre, Egypt	55	50 Anti-HCV antibody screen negative 5 control anti-HCV antibody positive
Department of Pathology Riyadh Armed Forces Hospital	1547	Blood Donors anti-HCV antibody 2 nd generation assay screen negative 693 for Saudi Arabian Nationals 134 from Sudanese Nationals 99 from Yemenis Nationals 621 from Egyptian Nationals
Brentwood Blood Donor Centre, UK	723	Control anti-HCV antibody negative UK Blood Donors

5.3.2.1 Cairo, Egypt

Dr A El-Zayadi from the Cairo Liver Centre sent 50 frozen serum samples from Egyptian blood donors which were known to be negative for anti-HCV antibody by second generation Ortho EIA and 5 control samples from anti-HCV antibody positive individuals to be tested with the genotype 4a core and NS4 antigens.

5.3.2.2 Brentwood Blood Donor Centre, UK

Murex Biotech obtained samples from 723 3rd Generation Ortho EIA anti-HCV screen negative UK blood donors, from the Brentwood Blood Donor Centre. The samples were used as controls to establish reasonable cut-off levels for the genotype 4 antigen assays. All 723 samples were tested with the genotype 4 NS4 antigen, 568 were tested with NS3 and 334 with genotype 4 core antigen.

5.3.2.3 Riyadh, Saudi Arabia

Dr AA Saeed of the Department of Pathology, at the Riyadh Armed Forces Hospital allowed a representative from Murex Biotech Ltd access to the blood donor samples at the hospital. Samples were available from 1547 second generation Ortho EIA screen negative individuals. The samples included 693 native Saudi Arabian individuals, 134 from immigrant workers from Sudan, 99 from Yemen and 621 from Egypt. Aliquots of samples were taken and sent to Murex Biotech for repeat analysis.

5.3.3 Assays

Samples	Laboratory	Test	Cut-off	Comments
Cairo Blood Donors	Edinburgh	single antigens	Non reactive O.D.< 0.2 Weakly reactive O.D, 0.2-0.6 Moderately reactive O.D, 0.6-1.0 Strongly reactive O.D, 1.0-2.0 +>2	
Saudi	Murex	Single antigens	Reactive if O.D 0.3 > background "noise"	> 0.25 obtain sample for repeat testing
Saudi	Murex	Single antigens Core	Positive>0.7	Mean of 80 samples plus 0.6O.D. units
Saudi	Murex	Single antigens NS3	Positive>0.78	Mean of 80 samples plus 0.6O.D. units
Saudi	Murex	Single antigens NS4	Positive> 0.898	Mean of 80 samples plus 0.6O.D. units

5.3.3.1 ELISA

ELISA plates had been coated with each of the commercial genotype 1b antigenic sequences for core and NS3. Proteins had been expressed from genotype 4a sequences to correspond to these antigens. These proteins were coated onto plates at similar concentrations and under similar conditions to generate tests with similar sensitivity and specificity (materials and methods section 2.6). The synthetic branched peptides used in the commercial serotyping ELISA from NS4 positions 1691-1708 and 1710 -1728 (Figure 4.1) (Bhattacharjee et al., 1995;Simmonds et al., 1993b) from both genotype 1b and 4a were also coated onto the solid phase of ELISA plates.

5.3.3.2 PCR

60 Aliquots of serum analysed in the Riyadh Armed Forces Hospital, from donors of Egyptian origin, which were reactive (10 seropositive in VK-48 assay and 50 with measured O.D.>0.25 in one or more single antigen plate), were sent to the Molecular Virology Laboratory for HCV PCR testing

5.3.4 Methods

The 55 samples from the Cairo Liver Centre, Egypt were tested by PCR (section 2.1), then the samples were tested in the core and NS4 single antigen plates of genotype 1b and 4a (section 2.6.1).

Murex obtained aliquots of serum from 723 UK seronegative blood donors from the Brentwood Blood Donor Centre. These samples were tested with the

genotype 4 core, NS3 and NS4 antigens as a comparison to the Saudi Blood Donors in order to determine cut-off levels.

A representative from Murex Biotech Ltd visited the Riyadh Armed Forces Hospital. Samples from 1547 individuals were screened with genotype 4a core, NS3 and NS4 antigens and 3rd generation anti-HCV screening assays.

The reactivity with the genotype 4 antigens of samples from 554 Ortho 3 EIA negative Egyptian and 898 Saudi/ Yemenis/ Sudanese was compared to the Brentwood Blood donors to establish cut-off levels. From this analysis of reactivity, samples which produced reactivity of greater than 0.25 O.D. units in one or more genotype 4 antigens were sent for further analysis. Aliquots of these samples were frozen and shipped on dry ice to Murex Biotech Ltd's office in Dartford for retesting with the genotype 4a antigen and comparable genotype 1b antigens. These sera were also tested with the commercial genotype 1b sequence Murex VK48 assay.

50 Samples from Blood Donors of Egyptian origin donated in Riyadh Armed Forces Hospital which were negative in the commercial third generation Ortho EIA but reactive with genotype 4 antigens were sent for PCR analysis. These were compared with 10 samples from commercial assay positive individuals.

5.3.5 Results

5.3.5.1 Cairo Samples

The samples from Cairo blood donors were tested in the genotype 4a and 1b Core and NS4 antigens. One of the negative samples was reactive with all antigens and was found to be PCR positive. On discussion with Dr El-Zayadi this was found to be wrongly identified as a negative sample. None of the other negative samples were PCR positive.

One sample was moderately reactive with the core 1b antigen (OD 0.816) and one sample had a weakly reactive reading of 0.417 with the genotype 4a NS4 antigen, but none of the other negative samples had elevated OD readings. The five control samples from HCV positive individuals were all PCR positive and were strongly reactive with all of the antigens. This demonstrates a high level of sensitivity and specificity for this assay.

5.3.5.2 Saudi Samples

Murex Biotech provided a table with the reactivity of genotype 4 antigens with Brentwood blood donors. Egyptian origin blood donors and Saudi/Sudanese/Yemenis blood donors. Also provided was a spread sheet with the original results of the genotype 4 antigens, the results of the repeated genotype 4 antigen assays, the results of assay with genotype 1b core, NS3 and NS4 antigens, the Ortho 3 EIA result, and VK48 ELISA results, for 132 sera from blood donors of Egyptian origin and 59 donors of Saudi/ Sudanese/ Yemenis origin.

5.3.6 Determination of cut-off value for genotype 4a antigen assays.

The reactivity of samples from Brentwood donor centre was compared with the initial data from the Saudi donor centre. In the core plates the reactivity of serum from 334 UK donors was compared with 554 donors of Egyptian origin, screen negative by Ortho 3 EIA, and 898 donations from Saudi, Yemen or Sudan nationals of “non Egyptian origin” (Table 5.2). In the Brentwood donor population 98.8% of donations had reactivity less than OD 0.3. This finding was similar to the reactivity observed with antigens in the Murex screening assay. Therefore the OD value 0.3 was considered the cut-off for background non-specific reactivity. An arbitrary plate cut-off value was assigned as the mean of 80 samples +0.6 OD units. The cut-off value given for core antigen was 0.7 units. Samples from individuals with reactivity of above this value would be considered as having genotype-specific reactivity to this antigen.

Analysis of the reactivity to the NS3 and NS4 antigens was carried out as above and any sample with a measured reactivity of greater than OD 0.3 was followed up. Cut-off values were assigned for NS3 and NS4, which were OD 0.78 and 0.898 respectively (Tables 5.3 and Table 5.4).

Range	Brentwood Donors		Saudi Donors –Egyptian Origin		Saudi Donors- Non Egyptian Origin	
	Frequency	%	Frequency	%	Frequency	%
0	0	0	0	0	1	0.11
0.1	272	81.44	255	46.03	560	62.36
0.2	54	16.17	251	45.31	296	32.96
0.3	4	1.2	26	4.69	30	3.34
0.4	2	0.6	6	1.08	6	0.67
0.5	1	0.3	8	1.44	1	0.11
0.6	0	0	0	0	0	0
0.7	0	0	2	0.36	0	0
0.8	0	0	2	0.36	2	0.22
0.9	1	0.3	2	0.36	0	0
1	0	0	1	0.18	0	0
2	0	0	1	0.18	0	0
3	0	0	0	0	2	0.22

Table 5.2 Summary Data for initial testing of the genotype 4a core antigen with Commercial HCV EIA seronegative blood donors.

Range	Brentwood Donors		Saudi Donors -Egyptian Origin		Saudi Donors- Non Egyptian Origin	
	Frequency	%	Frequency	%	Frequency	%
0	0		0		0	
0.1	280	0.00	150	0.00	276	0.00
0.2	246	49.30	367	27.08	581	30.73
0.3	28	43.31	23	66.25	25	64.70
0.4	7	4.93	6	4.15	4	2.78
0.5	3	1.23	1	1.08	3	0.45
0.6	2	0.53	1	0.18	2	0.33
0.7	0	0.35	0	0.18	2	0.22
0.8	0	0.00	0	0.00	0	0.22
0.9	0	0.00	2	0.00	1	0.00
1	0	0.00	1	0.36	0	0.11
2	0	0.00	2	0.18	1	0.00
3	2	0.00	1	0.36	3	0.11
		0.35		0.18		0.33

Table 5.3 Summary Data for initial testing of the genotype 4a NS3 antigen with Commercial HCV EIA seronegative blood donors.

Range	Brentwood Donors		Saudi Donors -Egyptian Origin		Saudi Donors- Non Egyptian Origin	
	Frequency	%	Frequency	%	Frequency	%
0	0	0.00	0	0	6	0.67
0.1	618	85.48	392	70.76	637	70.94
0.2	76	10.51	123	22.20	213	23.72
0.3	18	2.49	18	3.25	18	2.00
0.4	4	0.55	7	1.26	6	0.67
0.5	2	0.28	1	0.18	12	1.34
0.6	0	0.00	0	0.00	2	0.22
0.7	1	0.14	0	0.00	0	0.00
0.8	0	0.00	2	0.36	1	0.11
0.9	0	0.00	2	0.36	1	0.11
1	2	0.28	3	0.54	0	0.00
2	2	0.28	4	0.72	1	0.11
3	0	0.00	2	0.36	1	0.11

Table 5.4 Summary Data for initial testing of the genotype 4a NS4 antigen with Commercial HCV EIA seronegative blood donors.

5.3.7 Ortho 3 EIA

Murex personnel tested the blood donor samples from the Riyadh Armed Forces Hospital with the 3rd generation Ortho EIA. 33 anti-HCV positive donors were detected, 31 were of Egyptian origin, one of Sudanese origin and one of Saudi Arabian origin. 34 samples were not tested

5.3.8 Murex VK48

Two individuals whose samples were not tested by Ortho EIA were VK48 assay reactive. Another 4 individuals were VK48 assay positive alone.

34 individuals were not tested by Ortho 3 EIA. 11 were also not tested by Murex VK48 Assay due to sample volume. This included one sample which was repeatedly reactive with all 3 genotype 4 single antigens and the 3 genotype 1b single antigens which Murex also classed as a commercial positive.

5.3.9 Serology Results Single Antigens - Blood Donors of Egyptian Origin.

Of the 621 Saudi blood donors of Egyptian origin, data were available for 132 individuals. During testing in Saudi Arabia 125 samples produced OD values greater than O.D.0.25 to one or more genotype 4a antigens. The other 7 of the 132 cases where data were available included one donor who was borderline in the Ortho 3 EIA and two donors with genotype 4a core antigen reactivity on retesting in Dartford.

Genotype 4a directed reactivity was found in repeat testing in sixteen donors, however, only 3 of these provided measurements above the cut-off values set for each antigen (see Table 5.5). Reactivity to comparable genotype 1b antigens was observed in one of the donors reactive with the NS4 antigen and to the NS3 genotype 1b antigen in the individual reactive to both core and NS3.

Category	Saudi Blood Donors – Egyptian Origin		Saudi Blood Donor- Non-Egyptian Origin	
No Screened	621		926	
Ab>0.25 on one or more genotype 4a antigens	125 (20.1%)		58 (6.26%)	
No of samples retested	123		59	
Positive on one or more commercial 3 rd Gen EIA	34 (5.5%)		6 (0.65%)	
Borderline on one or more commercial 3 rd Gen EIA	7 (1.45%)		0	
Negative on 3rd Gen EIA Repeatable reactivity to genotype 4 a	O.D>0.3	O.D> χ +0.6	O.D>0.3	O.D> χ +0.6
Core Only	2 (0.32%)	0	1 (0.11%)	1 (0.11%)
NS3 only	7 (1.13%)	0	2 (0.22%)	0
NS4 Only	6 (0.96%)	2 (0.32%)	3 (0.43%)	0
Core and NS3	1 (0.16%)	1 (0.16%)*	1 (0.11%)	1 (0.11%)
Core and NS4	0	0	0	0
NS3 and NS4	0	0	0	0
Core, NS3, NS4	0	0	0	0

Table 5.5 The serological reactivity found in the Egyptian Blood Donors Tested on type 4a antigen.

*Reading above cut-off value for NS3 antigen only.

Repeatedly reactive samples OD χ +0.6 on table 5.6

5.3.10 Serology Results Single Antigens - Blood Donors of Saudi Arabian, Yemenis, and Sudanese Origins.

From the 926 samples, analyses data from 59 blood donors' serological assays were provided, of these 58 had measured initial test OD values greater than 0.25 units to one or more genotype 4a single antigens (leading to a sample being taken for repeat testing in Dartford) and one had a positive reaction with the VK48 assay. Two donors were Ortho 3 EIA positive, one of these was also VK48 positive and a further 4 donors were positive in VK48 alone. The donor positive with both Ortho and VK48 had positive results for all 3 genotype 4a single antigens as well as all three genotype 1b antigens. The donor only positive in Ortho 3 EIA was positive only in initial genotype 4a NS4 single antigen testing. One donor strongly positive in VK48 was not reactive with any genotype 4a single antigens, one VK48 reactive donor was repeatedly reactive with genotype 4a NS3 only, one was initially reactive with type 4 NS4 and the other repeatedly reactive with genotype 4a core and reactive with genotype 1b core.

Among the donors who were commercial serotyping assay negative, two individuals demonstrated reactivity greater than cut-off levels for genotype 4a antigens. One individual had reactivity towards the core antigen only and the other reactivity to genotype 4a core and NS3 antigens. No reactivity to the genotype 1b core was measured in the first individual, but the reactivity was also demonstrable to the equivalent antigens of genotype 1b in the second (see table 5.5). In the data provided there were no other cases of reactivity measured to genotype 1b antigens.

Sample	PCR	genotype 1 core	genotype 4 core	genotype 1 NS3	genotype 4 NS3	genotype 1 NS4	genotype 4 NS4
A	Negative	0.617	0.34	<u>2.336</u>	<u>3</u>	0.123	0.091
B	Negative	0.235	0.102	0.098	0.084	0.11	<u>1.687</u>
C	Negative	0.096	0.098	0.076	0.128	0.146	<u>1.084</u>
E	ND	0.133	<u>0.773</u>	0.062	0.064	0.067	0.081
F	ND	<u>2.649</u>	<u>2.649</u>	<u>3</u>	<u>2.846</u>	0.071	0.075

Table 5.6 Samples which were commercial assay unreactive, but which have genotype-specific reactivity with one or more single antigens.

Data shown in bold if >0.3 OD, shown in bold and underlined if > χ +0.6.

Samples A-C were from individuals of Egyptian origin, sample E and F are from individuals of Saudi origin

5.3.11 Reproducibility of the results

The results varied between initial testing in Saudi Arabia and testing in Dartford. For example 23 of the 72 donors who tested reactive with the genotype 4a core tested below the cut-off in the assay in Dartford. However, only 4 donors of the 40 donors who showed reactivity greater than the assigned cut-off value of 0.7 OD units, were below this value in repeat testing and only a further 3 donor reactivities were above the cut-off which were not above this value in initial testing (data not shown). Similarly for NS3 12 out of the 18 donors with reactivity were also reactive in repeat testing and no further reactivity above the cut-off value was discovered in repeat testing (see table 5.7).

The OD values for 179 samples for which both initial and repeat test values were available for all 3 antigens were used for analysis of the correlation between initial and repeat testing. The correlation between initial and repeat testing of the core antigen was 0.958, for NS3 it was 0.939 and 0.933 for NS4 (see figure 5.3).

Antigen	No. of Samples	Test in Saudi not reactive	Test in Saudi reactive
Core	Test in UK not reactive	110 (5 not retested)	23 (4 not retested)
	Test in UK reactive	4	45
NS3	Test in UK not reactive	124 (7 not retested)	23 (5 not retested)
	Test in UK reactive	6	26
NS4	Test in UK not reactive	105 (9 not retested)	25 (3 not retested)
	Test in UK reactive	6	45

Table 5.7 Frequency of reactivity of samples with single antigen tests in the field in Saudi Arabia and in the laboratory at Dartford.

Reactivity classed as OD >0.3

Reproducibility of the data generated for genotype 4 core antigen

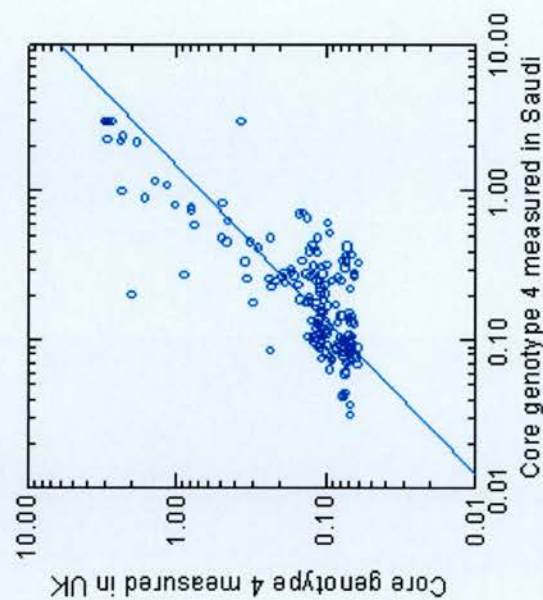


Figure 5.3 Correlation of results of genotype 4a core reactivity measured in Saudi Arabia compared to repeated reactivity results measured in Dartford

5.3.12 Reactivity to genotype 1b antigens

Reactivity to genotype 1b antigens was observed in most cases where reactivity to the genotype 4a antigen was detected. The only exceptions to this were in commercial assay positive donors where the specific reactivity, as measured by O.D. value, to the NS3 of genotype 1b was either higher than, or absent in, the genotype 4a antigen, and in one case where weak reactivity (OD 0.546) was observed in a donor with initial weak reactivity to genotype 4a NS4 (OD 0.305). The correlation between reactivity of donors, as measured in Saudi, for genotype 4a core to genotype 1b core was calculated to be 0.976 and 0.955 for the repeat testing in the UK. Similarly correlations of 0.713 –0.731 were observed for NS3 and 0.807 –0.845 for NS5.

Little information about genotype 1b reactivity in this population can be derived since the donors were initially selected on the reactivity to genotype 4a antigen.

5.3.13 Condition of samples during transit.

All samples of the Egyptian commercial assay negative group, that showed genotype 4a specific antigen reactivity, were PCR negative. These results were relayed to Murex Biotech Ltd personnel, who expressed concern about the storage conditions of the samples when they were transported from Saudi Arabia. It appeared the aliquots of serum were delayed in transit by customs and most of the samples arrived thawed, therefore the testing for HCV by PCR was severely hampered because the viral RNA in serum samples was likely to have degraded. To check the affect of the customs delay on the samples, 11 aliquots of serum which were positive with both the Ortho 3rd generation and Murex VK48 EIA assay were sent for PCR testing. Viral RNA was found in 7 of these samples (63% positive). This indicated it was likely that there had been some degradation of RNA, since results obtained by Murex Biotech indicated that in tests 80% of VK48 positive samples contain viral sequence (Dr B Rodgers personal communication).

5.3.14 Interpretation

The finding of genotype-specific serologically positive samples among the commercial assays negative group seemed to confirm the theory that samples were missed. However these samples were negative when tested for viral RNA and only one of these samples contained reactivity with more than one antigen. It is possible that the reactivity in these samples was residual from previous resolved infection, but it could also be attributed to cross reactivity to another antigen or to non-specific reactivity. The finding of genotype-specific reactivity to the genotype 1b antigens among the commercial assay positive and commercial assay negative populations of Egyptian origin was unexpected, since the predominant genotype of infection in Egypt was genotype 4. However, the Egyptian commercial positive samples, which were tested by PCR, contained 2 genotype 1 samples and 5 genotype 4 samples. Therefore, it was possible for genotype 1 specific reactivity to be present in these samples, in the same way that it is possible that genotype 4 infection was present in the non-Egyptian commercial assay negative individuals with genotype 4 specific reactivity. Saudi Arabia has a low prevalence of HCV in the native population and the most prevalent genotypes are types 1 and 2. The immigrant populations have left regions with high HCV prevalence and, therefore, one would expect the prevalence in the immigrants to be higher than the native population. The immigrants go to Saudi Arabia in search of work. Therefore, those infected with HCV would be more likely to be chronic asymptomatic carriers than patients with cirrhosis. The health care services in Saudi Arabia are very good and the frequency of new transmissions are low. Therefore, the HCV infections in this population were likely to be long established infections, where a number of individuals had recovered and several were recovering and clearing the virus. This would reflect the results obtained. The number of individuals who were HCV infected, and were positive in all assays, would represent the chronically infected individuals. The samples with high reactivity to a few antigens, including those of genotype 4, were representative of those infected several years ago who had since cleared the virus and now had a declining anti - HCV response.

- **Genotype-specific response was detected by non genotype one antigenic HCV sequence, however, it is likely that it detected a depleting immune response in resolving infection**

5.4 Screening Donor populations in Pakistan

5.4.1 Introduction

The failure to detect HCV PCR positive samples from infected individuals, which were reactive only with antigens expressed from sequence of the infecting virus genotype in Saudi Arabian blood donors, gave rise to wider study of blood donor populations. Part of the failure to find examples of PCR positive reactive samples with genotype-specific activity was attributed to the fact that the infection rate and disease prevalence in this population was low and response detected was likely to be from previous exposure. Therefore, the search for samples with genotype-specific reactivity was repeated in blood donations from a region where the infection rate was known to be much higher and the disease was much more prevalent. Dr Saeed Hamid of the Aga Khan University, Karachi, Pakistan confirmed from his studies that the prevalence of HCV was 7.5% and in some rural areas around Karachi was as high as 40% (Personal communication). The blood transfusion service reported that approximately 1 infectious unit of blood per 100 screened was entering the supply, despite screening with Abbott 2nd generation EIA. Dr Hamid also reported that the Liver Unit was experiencing some problems with HCV transmissions in their renal dialysis unit despite patient screening for HCV infection. Due to logistical problems it was not possible for samples from Saudi Arabia to be tested by PCR. However, the smaller number of samples studied from Pakistan could be PCR tested.

5.4.2 Samples

<u>Origin of samples</u>	<u>Number</u>	<u>Comments</u>
Karachi Blood Transfusion Centre	891	2 batches 405 all anti-HCV antibody negative, 3 anti-HBV antibody positive. 486 including 5 seropositive and 3 borderline in 2 nd generation anti-HCV antibody assay, 3 with elevated ALT results.

Dr Hamid sent 891 serum samples from the Karachi Blood Transfusion Service. The samples were sent in two batches. The first batch contained 405 samples, which were screened negative for anti-HCV antibodies, but these included three samples that were positive for anti-HBV antibodies. The second batch of 486 samples had also been screened for anti-HCV antibodies. However, this batch included five HCV seropositive samples and three samples that were borderline in the Abbott 2nd generation assay. Six samples which were positive for anti-HBV antibody and three samples with elevated ALT results were also included.

5.4.3 Assays

The samples were tested for reactivity in the Murex VK48 commercial genotype 1b screening assay and in equivalent genotype 3a antigens. The genotype 3a antigen assay plates were coated with a tripartite recombinant expressed protein of core, NS3 and NS5, and NS4 branched peptides from the serotyping assay (see section 2.6.2). The plates were coated at 200ng/well and were confirmed by Murex to be as near a match to VK48 in sensitivity as possible.

5.4.4 Methods

The serum samples were all tested in the Murex VK48 assay (section 2.7.5) and the BHC129 genotype 3a antigen test assay (section 2.6.2).

The first batch of samples were pooled and tested for HCV RNA by PCR of the 5' non-coding region. 50 µl of serum from five samples was mixed together in one 1.5ml Eppendorf tube and each pool of five samples had the RNA extracted using the Access RNA extraction method (section 2.1.1.8) and PCR performed (section 2.1).

Contamination of the primary PCR laboratory during testing of the second batch of samples resulted in only the samples that were reactive in the HCV ELISAs being tested by PCR.

The expense of ensuring consistent results was the main reason that only the seroreactive samples of the second batch were tested. The contamination was treated by swabbing the bench areas with 1% Hydrochloric Acid and soaking the end pieces of the Gilson pipettes in DNA and RNase-away solution (BRL Gibco, Paisley).

5.4.5 Results

All the samples were tested in the VK48 and BHC129 ELISAs. Thirteen samples had absorbance measured as greater than 0.600 OD units in both assays, 18 were reactive with the genotype 1b antigens only and 14 were reactive with the genotype 3a antigens only (see Table 5.8). PCR analysis was carried out, in pools of five, on all of the samples in the first batch. In the second batch of samples, due to contamination of the laboratory, PCR testing was only carried out on seroreactive samples. Of the thirteen samples that were reactive with both genotype antigens, seven samples were PCR positive. None of the samples, reactive in the VK48 assay or in the genotype 3 antigens alone, were found to be PCR positive. Additionally, there were no further PCR positive samples identified from the pooling of the first batch of samples. The samples, which were positive in both assays, contained 4 of the 5 seropositive and 1 of 3 of the borderline samples in the Abbott 2nd generation EIA. One anti-HBV antibody positive sample and a borderline Abbott 2nd generation result sample were positive in the genotype 1 assay, but not in the genotype 3 assay. The other Abbott 2nd generation positive and borderline samples, the other samples with anti-HBV antibody and the samples from individuals with elevated ALT levels were non-reactive in the assays.

Samples	Non-Reactive with Genotype 3a Assay		Reactive with Genotype 3a Assay	
	PCR negative	PCR positive	PCR negative	PCR positive
Non Reactive with Genotype 1b Assay	384*	0*	14	0
Reactive with Genotype 1b Assay	18	0	6	7

Table 5.8 Reactivity to Genotype 1b Commercial Assay and to the Genotype 3a Equivalent of the Commercial Assay in Pakistani Blood Donor Serum Samples.

Specific genotype 3a reactivity is shaded.

*Every sample was tested by PCR in the first batch of samples. Only reactive samples were tested by PCR in the second batch leaving 462 samples, which were not tested by PCR.

5.4.6 Interpretation

Eighteen samples were found to be commercial assay reactive with the genotype 1b sequence Murex VK48 assay during the testing in the Molecular Virology Laboratory. Only 5 samples were reactive and 3 samples borderline when tested in the genotype 1a sequence Abbott 2nd generation EIA in Pakistan. Seven samples were PCR positive, this included 3 of the Abbott 2nd generation positive samples. This indicated that a number of infectious samples (0.45% - 4 PCR positive samples from 883 with no anti-HCV antibody detected) were missed by screening in Pakistan, which would account for the reported infections despite commercial assay screening. However, the use of 3rd generation Abbott EIA assays should significantly decrease the rate of false negatives (Barrera et al., 1995).

There were 14 samples, which showed genotype-specific reactivity to the genotype 3a antigens, and 18 that were reactive with only the genotype 1b antigens (see Table 5.7). These samples with genotype-specific reactivity were negative when tested by PCR. The 14/891 (1.5% of samples), which contained reactivity to the genotype 3a antigens only, is similar to the 3/621 (0.48% of samples) of the commercial assay negative individuals of Egyptian origin, which contained reactivity to genotype 4a antigens. The reactivity, like that of the Saudi Arabian blood donors, could be the response to resolved infection or cross reactivity to another antigen or non-specific reactivity. The 18 samples that were reactive with the genotype 1b antigen alone could reflect the fact that the genotype 1b antigen was a better antigen. This was entirely possible since the genotype 1b antigens, as part of the commercial assay, have undergone many years of testing and refining. It may have also been possible that these individuals had been infected with genotype 1 infection that had resolved. Alternatively, the genotype 1b antigen may cross-react with other antibodies in the serum and give rise to false positives.

Genotype 3 is by far the most prevalent genotype in Pakistan, but genotype 1 infections do exist. The samples with elevated ALT and anti-HBV antibodies were not positive for HCV RNA nor anti-HCV antibody, which showed on this occasion, that screening for these surrogate markers of HCV infection did not improve detection. The overall finding was that detection of HCV seropositive samples was increased by 1.5% when screened by genotype 3a antigens compared with a 2nd generation screening assay.

- **Genotype-specific anti-HCV antibody response was detected in the blood donor population in Pakistan. However, genotype-specific response to genotype 1b was more frequently observed than response to genotype 3a.**
- **Detection of anti-HCV positive samples was increased by 1.5% when screened by 3rd generation compared to second generation screening assays.**
- **Detection was not improved by screening for surrogate markers of ALT elevation and anti-HBV antibody.**

5.5 Analysis of RIBA confirmation test results in Scottish Blood Donors

5.5.1 Introduction

The number of false positive anti-HCV results is high in a low HCV prevalence setting such as blood donor populations, where only 0.05% are infected (McOmish et al., 1993). Therefore, anti-HCV antibody positive samples found in EIA testing were retested in a second test before donors were recalled for investigation. The Recombinant Immuno-Blot Assay (RIBA) was used as confirmatory test of HCV infection by blood transfusion services. Each of the antigenic regions was coated separately and reactivity to each protein could be estimated. If serum reacted with two or more recombinant proteins, the sample would be classed as reactive. If the sample reacted with only one antigen, it was classed as indeterminate. Prior to PCR testing of all blood donations, the Molecular Virology Laboratory provided a HCV PCR service for all blood transfusion services in Scotland for PCR confirmation of samples from blood donors who were EIA positive, but were indeterminate in the RIBA 3rd generation assay. Frozen aliquots of these RIBA indeterminate serum samples were stored at -40°C in the Edinburgh laboratory.

The RIBA test contained bands from each of the HCV regions core (c22 peptide), NS3 (c33c expressed recombinant protein), NS4 (5-1-1p and c100p peptides) and NS5 (expressed recombinant protein). It also contains a band with superoxide dismutase protein and two bands of human IgG. The human superoxide dismutase (hSOD) band was used because the c33c and NS5 antigens were expressed as a fusion protein in combination with the superoxide dismutase gene (see Figure 1.9). The c33c recombinant protein was expressed

in *E.coli* and the NS5 antigen was expressed in *S.cerevisiae*. The RIBA hSOD band was used as a check to confirm that response detected against either of the expressed recombinant proteins was true anti-HCV reactivity and not reactivity directed against, or cross-reactive with, hSOD. The two human IgG bands were used for comparison to estimate the magnitude of the anti-HCV response. The amounts of protein coated on the two IgG bands were designated "level 1" and "level 2", the amount of reactivity to an antigen was assigned according to comparison with these bands.

The possible outcomes for activity of a RIBA band are:	
No band	
+/-	Inferior to level 1 IgG band, but a discernible band is present
1+	equal to the level 1 IgG band
2+	higher than the level 1 IgG band, but lower than the level 2 IgG band
3+	equal to the level 2 IgG band
4+	greater than the level 2 IgG band

The NS3 antigen in the RIBA test was expressed from a genotype 1a sequence amino acids 1192 to 1457. The distribution of HCV genotypes in the Scottish blood donor population was found to be 50% genotype 1, 10% genotype 2 and 40% genotype 3 (McOmish et al., 1994). The nucleotide sequence of the NS3 region varies considerably between the different genotypes; therefore some of the NS3 antigens may differ between genotypes. If individuals infected with virus of non-genotype 1 produced an antibody response directed against NS3 sequence of the infection genotype, then it is possible that the response may be genotype-specific and no response would be detected with genotype 1 sequence. If NS3 antigens of other genotypes were included in the RIBA assay, samples with genotype-specific responses may be detected. Perhaps samples, which were classed as RIBA indeterminates with core, NS4 or NS5 only bands reacting, could be resolved into RIBA confirmed cases by the use of NS3 antigens from other genotypes.

Samples which were RIBA indeterminate, but PCR positive, were rare after the introduction of 3rd generation screening and confirming assays. However two of these samples were obtained to test. In addition, blood donations which were found to be EIA positive and RIBA indeterminate, showed reactivity to the core, or NS4 or NS5 antigens alone, and were obtained in the first six months of 1995, were available to be tested for reactivity to NS3 of genotype 2b and genotype 3a.

5.5.2 Samples

	<u>Number</u>	<u>Comments</u>
Scottish Blood Transfusion Service	249	63 “core only” RIBA band positives blood donors 10 “NS4 only” RIBA band 82 “NS5 only” blood donors samples 94 anti-HBV, HCV and HIV antibody negative and HCV PCR negative blood donors

Serum samples from RIBA indeterminate Scottish blood donors, who donated in the first six months of 1995, were available for testing for reactivity to NS3 antigens of genotypes other than genotype 1. The samples consisted of: 63 “core only” RIBA band positives (8 samples scored 4+, 10 samples scored 3+, 29 samples scored 2+ and 16 samples scored 1+). Of these, 2 were HCV PCR positive (1 which scored 4+ and 1 which scored 2+); ten samples were “NS4 only” RIBA band (3 samples scored 4+, 5 samples scored 2+ and 2 samples scored 1+) and 82 “NS5 only” samples (8 samples scored 4+, 16 samples scored 3+, 32 samples scored 2+ and 26 samples scored 1+).

As a control group, 94 anti-HBV, HCV and HIV antibody negative and HCV PCR negative blood donors were also tested for HCV NS3 reactivity.

5.5.3 Assays

Assays	Interpretation of results
NS3 single antigen plate	Non-reactive O.D.< 0.2
Genotype 1b,	Weakly reactive O.D, 0.2-0.6
Genotype 2b	Moderately reactive O.D, 0.6-1.0
Genotype 3a	Strongly reactive O.D, 1.0-2.0

Each of the RIBA indeterminate samples was tested in ELISA plates coated with NS3 antigen of either genotype 1b, 2b or 3a sequence. The genotype 1b sequence was the DX458T1 sequence from amino acids 1360 to 1454, which was used in commercial assays. The genotypes 2b and 3a sequence were the expressed protein from the corresponding portion of the genotype 2b and 3a genome, which had been amplified and cloned from samples LJ823 and LJ516. The genotype 2b NS3 clone, DX502, and genotype 3a NS3 clone, DX506, were expanded and the protein expressed in TOP10 cells at Murex Biotech Ltd and coated onto ELISA plates at a concentration of 25-50 ng per well as described in section 2.5.

5.5.4 Methods

All of the indeterminate samples were tested for reactivity with antigen of NS3 of genotype 2b and 3a sequence. The NS3 genotype 1b antigen coated plates were in short supply; therefore only 22 of the c22 (core) antigen only indeterminate samples and the 94 control negative blood donors were tested for reactivity with this antigen. The control blood donors were not tested for reactivity to genotype 2b antigen. Each sample was tested by adding 20 µl of the thawed and homogenised serum to 180 µl of serum diluent and pipetted into a well of the ELISA plate. Each plate contained 2 wells with 20 µl aliquots of control positive sample and 3 wells of control negative samples. Both positive and negative samples used were the control samples supplied with the Murex VK48 commercial assay. The assay was carried out as described in the protocol for a Murex VK48 screening assay as described in section 2.6.1.

5.5.5 Results

The HCV PCR positive RIBA core reactive 4+ sample was not reactive with any of the NS3 antigens and the RIBA core reactive 2+ sample was not reactive with the NS3 genotype 2b or 3a antigens and was not tested with the genotype 1b antigen.

The 94 control blood donors were also unreactive in the NS3 genotype 1b and only 3 samples were weakly reactive in NS3 genotype 3a antigen plates (see Table 5.9).

No reactivity was measured with the genotype 1b NS3 antigen, in any of the 116 samples tested.

Five samples were identified which reacted strongly with the non-genotype 1 NS3 antigens, two samples which reacted with genotype 2b and 3a, two which reacted with genotype 2b only and one which reacted with genotype 3a only. Three samples (one sample core score 3+, one sample core score 2+ and one sample NS5 1+) were strongly reactive (absorbance >1 OD units) with the NS3 genotype 3a antigen. The core reactive samples were also strongly reactive on the NS3 genotype 2b antigen, but not on the NS3 genotype 1b antigen. The NS5 reactive sample was not reactive on the genotype 2b antigen and was not tested on genotype 1b antigen.

A further two samples (a core score 1+ and a NS5 score 2+) were strongly reactive in the NS3 genotype 2b antigen plates but not the genotype 3a plates. The c22 reactive sample was not reactive with genotype 1b antigen and the NS5 reactive sample was not tested with antigen of NS3 genotype 1b.

Two samples (both c22 score 3+) were moderately reactive (absorbance 0.6 - 1.0 OD units) on the genotype 3a NS3, both were weakly reactive (absorbance 0.2-0.6 OD units) on NS3 genotype 2b (0.423 and 0.36 OD units), but not reactive on genotype 1b antigen. A further eleven samples (one c22 2+ and three NS5 3+) had weak reactivity with genotype 2b NS3 antigen, but no reactivity against genotype 3a antigen was detected. Seventeen samples showed weak reactivity with NS3 of genotype 3a. Only three of these (c22 reactive samples) were also weakly reactive with the genotype 2b NS3 antigen.

Antigen / reactivity	Core Indeterminate	NS4 Indeterminate	NS5 Indeterminate	Negative Blood Donors
NS 3 Genotype 1b				
# Reactive / # tested	0/22	0/0	0/0	0/94
Weakly reactive	ND	ND	ND	ND
Moderately reactive	ND	ND	ND	ND
Strongly reactive	ND	ND	ND	ND
NS 3 Genotype 2b				
# Reactive / # tested	12/63	1/10	7/82	0/0
Weakly reactive	9	1	6	0
Moderately reactive	0	0	0	0
Strongly reactive	3	0	1	0
NS3 Genotype 3a				
# Reactive / # tested	12/63	1/10	4/82	3/94
Weakly reactive	8	1	3	3
Moderately reactive	2	0	0	0
Strongly reactive	2	0	1	0

Table 5.9 The Reactivity of RIBA Indeterminate Samples for Genotype 1b, 2b and 3a NS3 Antigen.

Weakly reactive was classified as any OD measured between 0.2 and 0.6 OD units, Moderately reactive was classified as any OD measured between 0.6 and 1.0 OD units, Strongly reactive was classified as any OD measured between 1.0 and >2.0 OD units

The reactive samples were retested for HCV by PCR using amplification primers for the 5' non-coding region. None of the samples were PCR positive, although the control dilutions series confirmed positive results to a dilution containing 1×10^4 copies/ ml.

The eleven samples with the highest NS3 reactivity were tested by the Murex NS4 serotyping assay. None of these samples had detectable NS4 antibody, therefore no serotype could be determined.

5.5.6 Interpretation

Reactivity to only one antigen suggests that the reactivity is likely to be cross-reactive or non-specific. Therefore, many of these RIBA indeterminate samples are likely to be real false negatives. However, it is possible that some of these results were a genuine response to a previous, or newly acquired, HCV infection, as is the case for the two PCR positive samples. The immune response may only be targeted to a single antigen because the response is just being mounted or is declining after the infection is resolved.

The RIBA assay, like the commercial screening assays, is also based on a genotype 1a sequence. It is likely that the response detected in individuals with non-genotype 1 infection is also reduced by between 2- to 5- fold. The use of antigens with sequences from the other HCV genotypes may resolve indeterminate sample results by detecting genotype-specific reactivity. If the indeterminate samples have a weak response, an antigen of the infecting genotype will better detect this.

Five samples were identified which reacted strongly with the non-genotype 1 NS3 antigens, two samples which reacted with genotype 2b and 3a, two which reacted with genotype 2b only and one which reacted with genotype 3a only. Each of these samples also has anti-HCV antibodies to another region other than the NS3 as detected by RIBA. The antibody in these samples may be evidence of previous HCV infections. The lack of detectable antibody to the NS4 region prevented detection of a serotype, by competitive ELISA (section 2.7.5), which in the absence of detectable virus by PCR could have proved infection with a non-genotype 1 virus.

- Use of NS3 antigens of non-genotype 1 did not allow RIBA “confirmation” of the two true infection cases (PCR positive samples).
- Use of the NS3 antigens “confirmed” specific anti-HCV antibody in 5 RIBA unconfirmed cases; these may represent resolved infection.

5.6 Serological Diagnostic Test Results in Hepatitis Patients from Pakistan

5.6.1 Introduction

PCR positive samples that were commercial screen assay negative, but reactive with antigens of the infecting virus genotype, could not be found in blood donor populations. The prevalence of infection is lower in blood donors, therefore, these samples might be found in populations with a higher prevalence of infection. This led to the direct search for these samples in cryptogenic hepatitis patient populations. If samples, from patients in regions where other virus genotypes were prevalent, were under-detected in commercial assays, then investigation into patients diagnosed with non A-E hepatitis should yield true HCV positive infections. In addition, patients with other forms of viral hepatitis may also have undiagnosed HCV co-infection, due to false negative anti-HCV results.

Serum samples were made available from patients attending the Liver Unit in The Aga Khan University Hospital in Karachi, Pakistan. The cryptogenic hepatitis patients' samples and those with HBV infection were to be tested by PCR for HCV RNA and by ELISA for reactivity to NS3 antigen expressed from genotype 3a sequence. If type-specific reactivity to genotype 3, the predominant genotype of infection in Pakistan, affects commercial assay results then the use of genotype 3a NS3 antigen may help resolve false negative results.

5.6.2 Samples

	<u>Number</u>	<u>Comments</u>
Aga Khan University Hospital Liver Unit	160	41 cryptogenic hepatitis 25 fulminant hepatitis negative for anti-HCV (on 2 nd generation assay) and HBV antibody, 17 anti-HBV antibody positive but anti- HCV antibody negative, 69 anti-HBV antibody negative but anti-HCV antibody positive and 8 anti- HCV and HBV antibody positive

Dr Hamid had previously sent frozen serum samples from 160 individuals to the Molecular Virology Laboratory in Edinburgh. These hepatitis patient samples included: 41 with cryptogenic hepatitis and 25 with fulminant hepatitis, which were anti-HBV and HCV antibody negative; 17 samples which were anti-HBV antibody positive and anti-HCV antibody negative; 69 samples which were anti-HBV antibody negative and anti-HCV antibody positive; and 8 samples which were anti-HBV and HCV antibody positive.

5.6.3 Assays

Assays	Interpretation of results
VK48 Murex	Manufacturer's instructions section 2.7.1
Chiron RIBA	Manufacturer's instructions section 2.7.4
Genotype 3a single antigen	Non-reactive O.D.< 0.2 Weakly reactive O.D, 0.2-0.6 Moderately reactive O.D, 0.6-1.0 Strongly reactive O.D, 1.0-2.0 +>2
BHC129 genotype 3a multiple antigen plates	

The samples were tested for anti-HCV antibody by the laboratory in Pakistan with the Abbott 2nd generation assay. Information about the HBV antibody testing in Pakistan was not available. When the serum samples arrived in the Molecular Virology Laboratory in Edinburgh the samples were allowed to defrost and were tested by PCR for HCV RNA using primers for the 5' non-coding region. (See methods section 2.1)

The samples were tested in the commercial assay VK48 from Murex Biotech Ltd. as directed by the manufacturer's instructions described in section 2.7.1. Several samples were rechecked in the Abbott 2nd generation assay by Mr P McCulloch of the Hepatitis Reference Laboratory. Samples that were of interest were also tested in the Chiron RIBA 3rd generation assay, which was carried out according to the manufacturer's instructions as described in section 2.7.4. Murex Biotech Ltd supplied ELISA plates, which had been coated with antigen that they had expressed from the clone DX506, which was constructed from an expression clone containing the cDNA sequence of NS3 from a genotype 3a sample.

The ELISA with the NS3 genotype 3a antigen was carried out as described in section 2.6.1 using reagents supplied by Murex Biotech and the absorbance in the wells measured at 470 nm. Discrepant samples (sample numbers 348, 349, 514 and 515) were tested in the BHC129 assay, which contained the expressed sequence from genotype 3a core, NS3 and NS5 with NS4 synthetic peptides derived from genotype 3 sequence. This assay was carried out as described in section 2.6.2.

5.6.4 Methods

The HCV PCR status of the samples was available from a previous study on these samples. Of the 41 samples from cryptogenic hepatitis patients, which were anti-HBV, and anti-HCV antibody negative, 31 were PCR negative, 9 were PCR positive and one sample had insufficient volume to test. Of the 25 fulminant hepatitis patient samples, which were anti-HBV and HCV antibody negative, 24 were PCR negative and 1 contained insufficient volume to test. Of 17 samples that were anti-HBV antibody positive and anti-HCV antibody negative, 12 were PCR negative, 4 were PCR positive and one had insufficient volume to test. The 69 samples, which were anti-HBV antibody negative and anti-HCV antibody positive, consisted of 15 PCR negative, 51 PCR positive and 3 samples with insufficient volume to test. Of the 8 samples, that were anti-

HBV and HCV antibody positive, 5 were PCR negative, 1 was PCR positive and 2 contained insufficient volume.

Initially, an equal number of PCR positive and PCR negative samples from each of the non-HBV and HCV patients (5 samples), the HBV patients (2 samples) and HCV patients (7 samples) were selected and assessed for NS3 genotype 3a reactivity in the single antigen plates. Encouraging results showed reactivity with genotype 3a NS3 in PCR positive samples which were Abbott 2nd generation negative (Table 5.10). To check for false negatives arising from the Abbott 2nd generation assay, all samples with anti-HCV antibody negative results were tested in the commercially available Murex VK48 3rd generation screening assay. Identified samples which were positive in the VK48 and/or PCR positive were sent to be retested by the Abbott 2nd generation assay in the Hepatitis Reference Laboratory and were also tested in the RIBA 3rd generation assay. The genotype of five of the samples of interest was determined by RFLP of an amplified sequence from the 5' non-coding region as described in section 2.1.2.1

5.6.5 Results

5.6.5.1 NS3 genotype 3a reactivity.

Of the cryptogenic hepatitis anti-HCV and anti-HBV antibody negative, but HCV PCR positive samples, 3 of the 5 samples showed reactivity (O.D. > 0.2) to the HCV genotype 3a NS3 antigen compared with none of the 5 HCV PCR negative samples. All of the seven HCV PCR positive anti-HCV antibody positive, but anti-HBV antibody negative, samples contained strong reactivity (O.D. 1.0-2.0) to the HCV genotype 3a NS3 antigen, compared with only 4 of the PCR negative samples, which showed strong reactivity and one sample with moderate reactivity (O.D. 0.6-1.0; measured absorbance 0.627). Of the anti-HBV antibody positive and anti-HCV antibody negative samples, one of the two HCV PCR positive samples contained reactivity to the HCV genotype 3a NS3 antigen, but neither of the HCV PCR negative samples displayed any reactivity (see Table 5.10).

Patient Group	Number of Patients Tested	Number of Patients with Reactivity to NS3 Antigen of Genotype 3a
HBV negative / HCV negative / PCR positive	5	3
HBV negative / HCV negative / PCR negative	5	0
HBV positive / HCV negative / PCR positive	2	1
HBV positive / HCV negative / PCR negative	2	0
HBV negative / HCV positive / PCR positive	7	7
HBV negative / HCV positive / PCR negative	7	5

Table 5.10 Reactivity to NS3 Antigen of Genotype 3a Among Liver Unit Patients, which were Serum RNA Negative or Serum RNA Positive. Reactivity defined as an O.D of greater than 0.6

5.6.5.2 Commercial Assay based on genotype 1b sequence (Murex VK48) results.

All the samples with anti-HCV antibody results from the Abbott 2nd generation assay in Pakistan were tested in the Murex VK48 commercial assay (see Table 5.11). The serum from the cryptogenic hepatitis anti-HCV and anti-HBV antibody negative cohort (41 individuals) gave 9 positive results, 31 negative results and one sample contained insufficient volume to test. The 9 positive results included seven of the HCV PCR positive samples. The other two HCV PCR positive samples were negative in the VK48 assay. All of the 25 fulminant hepatitis patient samples were negative in the VK48 assay. Of the 17 anti-HBV antibody positive, but anti-HCV antibody negative group, 2 samples tested positive in the VK48 assay, 14 samples tested negative and one sample had insufficient volume to test. Two of the four PCR positive samples in this group were among the VK48 positive results.

Patient Group	Number of patients	PCR status	VK48 Result
Cryptogenic hepatitis HBV negative / HCV negative	<u>41</u>	31 negative 9 positive 1 insufficient to test	31 negative (2 PCR positive) 9 positive (7 PCR positive) 1 insufficient to test
Fulminant hepatitis HBV negative / HCV negative	<u>25</u>	24 negative 1 insufficient to test	25 negative
HBV positive / HCV negative	<u>17</u>	12 negative 4 positive 1 insufficient to test	14 negative (2 PCR positive) 2 positive (2 PCR positive) 1 insufficient to test
HBV negative / HCV positive	<u>69</u>	15 negative 51 positive 3 insufficient to test	ND
HBV positive / HCV positive	<u>8</u>	Negative 1 positive 2 insufficient to test	ND

Table 5.11 HCV PCR Status and Murex VK48 Commercial Assay Results Among Liver Unit Patients and Blood Donor Controls from Karachi.

5.6.5.3 Discrepant Samples

All samples with a PCR positive result and/or a positive VK48 were retested in the Abbott 2nd generation assay (with the exception of sample 515 which could not be located), RIBA 3rd generation and in the genotype 3a NS3 antigen.

The retesting by Abbott 2nd generation assay in Edinburgh, found 5 samples had been falsely identified as negative in Pakistan (samples 82, 521, 566, 582 and 496) and another sample was found to be borderline in the test (sample 349) (see Tables 5.12 and 5.13). Of the five false negative samples, four were HCV PCR positive. The sample, which was borderline in the test, was also PCR positive. The Abbott 2nd generation result differed from the Murex VK48 result in six instances (samples 349, 480, 514, 281, 531 and 557) with the VK48 result reflecting the PCR result in all but one of the samples (281).

RIBA results detected anti-HCV antibody in all but 3 of the discrepant samples (numbers 515, 34 and 78). Low levels of c100 and c33c reactivity were detected in sample 348, which was not detected by Abbott or Murex tests.

Sample	PCR status	Genotype	Abbott EIA (2 nd Gen)	VK48	NS 3 Type 3	Genotype 3a EIA	RIBA c100	RIBA c33c	RIBA c22	RIBA NS5
82	POS	3	POS	POS	>2	ND	4	4	4	4
348	POS	ND	NEG	NEG	0.094	0.097	1	1	0	0
349	POS	3	IND	POS	0.036	1.814	3	1	3	1
480	POS	3	NEG	POS	>2	ND	4	3	0	3
514	POS	ND	NEG	POS	0.75	1.845	1	1	3	0
515	POS	3	ND	NEG	0.015	0.146	0	0	0	0
521	POS	ND	POS	POS	>2	ND	4	4	4	1
566	POS	ND	POS	POS	>2	ND	4	4	4	4
582	POS	3	POS	POS	>2	ND	3	3	4	4
281	NEG	-	NEG	POS	0.366	ND	1	1	0	2
496	NEG	-	POS	POS	0.771	ND	2	3	4	2

Table 5.12 Discrepant Samples from Liver Unit Patients found to be Anti-HBV and Anti-HCV Antibody Negative by Testing in Pakistan.

Abbott 2nd generation results are the retest results from the Hepatitis Reference Laboratory in Edinburgh, PCR positive samples that are commercial assay negative are shown in bold print.

Sample	PCR status	Genotype	Abbott EIA (2 nd Gen)	VK48	NS Genotype 3a	3	Genotype 3a EIA	RIBA c100	RIBA c33c	RIBA c22	RIBA NS5
34	POS	ND	NEG	NEG	<u>0.518</u>		ND	0	0	0	0
78	POS	ND	NEG	NEG	0.085		ND	0	0	0	0
531	POS	ND	NEG	POS	>2		ND	4	4	4	4
557	POS	ND	NEG	POS	>2		ND	4	3	4	1

Table 5.13 Discrepant Samples from Liver Unit Patients found to be anti-HBV antibody positive and anti-HCV antibody negative by testing in Pakistan.
 PCR positive samples, which are commercial, assay negative, are shown in bold print. The NS3 genotype 3a antigen result for sample 34 is highlighted because it is the only reactivity measured in this sample.

5.6.5.4 Genotype 3 NS3 Reactivity.

Genotype 3a NS3 reactivity was detected strongly (absorbance O.D. 1.0- 2.0 + >2) in seven and moderately in two (absorbance O.D. 0.6- 1.0) (514 and 496) of the discrepant samples with weak reactivity above baseline (O.D. 0.2-0.6) in a further two samples (281 and 34). Reactivity in samples 348, 349, 514 and 515 was tested in the genotype 3a multiple antigen assay. Unfortunately, none of samples 34 and 78 remained to test in this assay. The genotype 3a assay detected reactivity in sample 349 and 514, but not in samples 348 and 515.

5.6.6 Interpretation

The Abbott 2nd generation testing in Pakistan is generating false negatives. The error rate in the cryptogenic hepatitis anti-HBV and HCV antibody negative group was 12%. Five out of the forty-one samples were falsely reported as negative. The false negative results may occur because of poor sample preparation or storage or may be due to operator error in the testing laboratory. Use of a 3rd generation commercial screening assay may improve the detection of HCV infections. The VK48 screening assay was much better at detecting infectious samples, but 4 infections (samples 348, 515, 34 and 78) were identified which could not be detected by serology. One of these samples (sample 34) was weakly reactive with recombinant antigen of NS3 genotype 3a (0.518 OD units). Unfortunately, none of this sample remained to be tested in the genotype 3a antigen ELISA. The samples, which were PCR positive and reactive in both Abbott and Murex tests, were strongly reactive with the genotype 3a NS3 antigen. The genotype 3a NS3 antigen reactivity was lower for samples that were Abbott negative or PCR negative, from the group of individuals without markers for HBV co-infection.

Of the samples that were not detected by the screening assays, one sample (348) had weak reactivity (1+) to the c100 and c33c RIBA antigens. These antigens correspond to the NS3 and NS4 regions, which are highly variable. Unfortunately it was not possible to test the sample for genotype 3 NS4 reactivity. The genotype 3 NS3 antigen did not detect the NS3 reactivity detected by RIBA. The genotype of infection was not known. It is possible, although unlikely, that the sample was from an individual with genotype 1 infection and the reactivity was genotype-specific and this was the reason no reactivity was measured with the genotype 3 NS3 recombinant antigen. It is

also possible that the RIBA NS3 antigen is a better antigen and detects response much better than the recombinant genotype 3 NS3 antigen.

Two samples (348, 281) that were negative by Abbott 2nd generation, did not have any detectable anti-core response in the RIBA test. The core antigen is the most conserved between genotypes and the most cross-reactive response was measured with this antigen. The negative results with the Abbott 2nd EIA may have been because the antibody response to the NS3 and NS4 regions was genotype restricted and did not cross-react with the assay antigens.

- **The study of the liver unit patients from Pakistan has identified two problems with screening for HCV by 2nd generation assay. One is that the number of false negatives is high; the other is that there are a number of patients who do not have a detectable antibody response.**
- **The use of genotype 3 antigens in this group would not have improved the detection of HCV in this population.**

5.7 Seroconversion Panels

5.7.1 Introduction

Seroconversion panels are sequential samples taken from an infected individual over a time period, during which the immune response is mounted and becomes detectable by serological assay. Some of these seroconversion panels have been obtained, in the past, from blood transfusion services, which pay for donations. In these centres drug addicts often made frequent repeated donations to help fund their habit. This allowed study of the immune response in individuals contracting infections. Large volumes of serum from different time points since infection would be available for analysis. Other seroconversion panels were obtained from monitoring of individuals who were accidentally infected and “look back” studies on people who received infected blood or blood products. The seroconversion panels were valuable tools for diagnostic research as these samples allowed the efficiency of assays to be tested. Seroconversion panels were a convenient way to test the assays with antigens from non-genotype 1 sequence. The seroconversion may occur earlier with antigens from the infecting genotype than with conventional assays due to type-specific immunity.

Samples from individual J were described as a seroconversion panel; however, this patient already had detectable response in the initial sample. This patient cleared HCV infection after IFN therapy. The serology results are included in this section to demonstrate the extent of the type-specific response in the resolving infection and declining immune response.

5.7.2 Samples

<u>Samples</u>	<u>Number</u>	<u>Comments</u>
Seroconversion genotype 3a panels	10	3 acute hepatitis patients 1 infected donor and 2 recipients 4 no information
Control	10	4 negative controls 5 commercial anti-HCV antibody positive and PCR positive samples 1 window period

Dr A Widdell of Malmo University, Malmo, Sweden provided serum from 10 individuals infected with genotype 3a virus, where 2 or more sequential samples were available. These samples came from individuals A - J. A, B and C were patients with acute hepatitis (2 samples were available for each of patients A and B, and 3 samples from patient C). Individual D was a HCV positive blood donor, whose antibody negative blood donation had been used in the treatment of individuals E and F (3 samples were available for individual D and 2 each for individuals E and F). No further information is known of individuals G, H, I and J other than they were infected with genotype 3 virus (3 samples are available for individuals G, H and I and 12 samples were obtained from individual J). A further 10 control samples were included, which contained 4 PCR and serologically HCV negative blood donor samples, 5 samples with antibody positive and PCR positive results (1 mixed genotype 1b and 2b infection, 1 of genotype 2b viral infection, 2 of genotype 4 viral infection, 1 of genotype 6a) and 1 sample where the only information available was that the individual was in the window period of infection with HCV virus.

These samples had been extensively studied in the laboratory at Malmo University, Abbott 2nd generation EIA and IMX tests, 3rd generation RIBA, (3rd generation Ortho EIA had been performed on sample J) PCR and RFLP had been performed. Only a small volume of sera could be spared for further testing. Initially 50 µl aliquots of the frozen sera from patients A-J were sent under blinded code for analysis with NS3 genotype 1b and genotype 3a NS3 antigen assay. A further 30 µl frozen aliquot of each sample was sent unblinded for analysis with the BHC129 genotype 3a multiple antigens ELISA. PCR test results were not available for seroconversion samples G, H and I. With only 50 µl and 20 µl samples available for analysis, it was not possible to confirm the PCR status of these samples.

5.7.3 Assays

The NS3 genotype 1b and 3a recombinant antigen coated plates were used initially for the coded samples and, at a later date, the uncoded samples were tested in BHC129, the genotype 3a combined antigen assay. Murex Biotech Ltd provided ELISA plates coated with recombinant protein from NS3 region amino acid 1360 to 1454, from the commercial assay genotype 1b, obtained from DX458 sequence and the genotype 3a recombinant protein expressed from the clone DX506. BHC129 assay wells were coated with a tripartite fusion protein of core NS3 and NS5 as previously described section 2.6.2. Murex Biotech Ltd supplied sample diluent, goat anti-human IgG conjugate, substrate and wash buffer and 0.5M sulphuric acid was made up in the laboratory. The previously described negative control obtained from the SNBTS was used in 2 wells of each assay and the high antibody titre anti-HCV positive sample was used as a positive control in 2 wells.

5.7.4 Methods

The frozen samples shipped from Malmo University were allowed to thaw and were mixed gently by pipette, before 20 µl of each sample was added to the respective well of each assay plate. The assays were carried out as described in section 2.6.2 and the results expressed as the optical density absorbance at 470 nm in the substrate wells after the addition of 0.5 M sulphuric acid. The results of the samples, which were received under code, were faxed to Dr Widdell, who faxed back the key to which code matched which sample.

The PCR status for each of the samples from individual G, H and I was not

available. Therefore, it was not known if the patient was infected at the time of the first two samples for each individual. Unfortunately, no further information was available about these individuals. Assuming these samples were PCR positive at the time of the first sample, it would be very interesting if patient I had remained serologically negative for 8 years.

Unfortunately, Dr Widdell did not provide the cut-off values for the Abbott 2nd generation EIA and IMX assays and 3rd generation EIA tests.

5.7.5 Results

5.7.5.1 Individual A

The 14/09/94 sample was reactive with both the BHC129 assay and the Abbott 2nd generation EIA. No reactivity was measured in the genotype 1b and genotype 3a NS3 antigen coated plates. The RIBA test indicated reactivity to the core antigen. However, since this was the only antigen reactive in this test, the sample would be classed as indeterminate. In the sample of the 25/10/94 the IMX and RIBA results were both positive, as was the BHC129 and Abbott 2nd generation assays, but neither of the single NS3 antigens tests were reactive, although reactivity to the RIBA C33c antigen suggests NS3 reactivity. There was insufficient volume of the sample from 14/12/94 left for testing.

5.7.5.2 Individual B

The 01/09/94 sample was not detected by either of the NS3 antigen coated plates, but was strongly reactive in the genotype 3a BHC129 assay and also in the Abbott 2nd generation EIA but was RIBA indeterminate (c22c only). The 12/10/94 sample was PCR negative; however, antibody was detected in all assays. The genotype 3a NS3 antigen test measured an OD of > 2 compared with an OD of 0.789 in genotype 1b assay.

Individual	Date of Sample	Abbott Generation EIA	Abbott Generation IMX	2 nd	RIBA 3**	PCR Genotype	/	NS3 Genotype 1b	NS3 Genotype 3a	BHC129 Assay
A	14/07/94	2.67	0.63, 0.79		0,0,3,0	POS 3a		0.058	0.018	>2
A	25/10/94	2.78	1.69		1,3,1,0	POS		0.045	0.102	>2
A	14/12/94	4.02	2.16		4,4,1,0	NEG		NA	NA	NA
B	01/09/94	1.8	0.76, 1.04		0,0,4,0	POS 3a		0.015	0.024	>2
B	12/10/94	2.09	4.21		0,4,4,0	NEG		0.789	>2	1.998
C	21/07/94	3.38	0.76, 0.87		0,4,4,0	POS 3a		0.033	0.018	NA
C	29/07/94	3.4	0.74, 0.84		0,4,4,0	POS		0.027	0.028	NA
C	29/09/94	4	6.09, 8.95		0,4,4,0	NEG		0.027	0.354	>2

Table 5.14 The Serological Results for the Seroconversion Panel of Samples from Individuals A, B and C.

The reactivity to RIBA antigens is listed in C100, C33c, c22 and NS5 order.

5.7.5.3 Individual C

In individual C, the EIA positive result was RIBA confirmed in each of the 3 samples. The NS3 single antigen plates did not detect any antibody response. However, in the 29/09/94 sample, the OD in the genotype 3a NS3 antigen test (0.354) was higher than that of the genotype 1b antigen test and elevated above background readings. Insufficient volume of the 21/07/94 and 29/07/94 samples prevented testing in the BHC129 assays. The 29/09/94 sample was strongly reactive in the BHC129 assay (Table 5.14).

5.7.5.4 Individual D

The blood donor's first sample (05/10/93) had no detectable antibody by any commercial assay, the Abbott IMX measurement of 0.5, being the only elevated value. The sample of 03/11/93 was positive by EIA 2 and IMX and confirmed by RIBA. This sample was also detected by the BHC129 assay (OD >2). The NS3 genotype 3a antigen detected low levels of NS3 antibody (0.557) compared with none in the NS3 genotype 1b assay (0.025). The third sample (27/10/94) was slightly detected by NS3 genotype 1b antigen (0.46) compared with strong detection (>2) in the NS3 genotype 3a antigen and the BHC129 assay.

5.7.5.5 Individual E

The two samples are 2nd generation EIA and IMX positive and RIBA assay confirmed, although the patient has undergone interferon treatment and cleared the virus to become PCR negative. Both samples are strongly reactive with the BHC129 and NS3 genotype 3a assays compared with low reactivity to the NS3 of genotype 1b recombinant protein antigen (0.223 and 0.227 first and second samples respectively).

5.7.5.6 Individual F

This is the second recipient of the infected blood / blood product from individual D. Again, both samples were Abbott EIA and IMX 2nd generation positive and RIBA confirmed. The 13/04/94 sample was PCR negative. Both samples were strongly reactive in the BHC129 assay. The genotype 3a NS3 antigen assay measured a much greater response than the genotype 1b NS3 antigen (1.427 and >2 compared with 0.165 and 0.167 for the 18/01/94 and the 13/04/94 samples respectively) (Table 5.15).

Individual	Date of Sample	Abbott Generation EIA	Abbott Generation IMX*	2 nd	RIBA 3**	PCR Genotype	NS3 Genotype 1b	NS3 Genotype 3a	BHC129 Assay
D	05/10/93	NEG	0.5		0,0,0,0	POS 3a	0.005	0.006	0.071
D	0/11/93	2.21	3.21		0,4,3,0	POS	0.025	0.557	>2
D	27/01/94	3.97	11.3		NA	NA	0.46	>2	>2
E	10/01/94	4.94	9.7		2,3,4,0	POS 3a	0.223	>2	>2
E	20/04/94	11.9	11.9		2,4,4,0	NEG	0.227	>2	>2
F	18/01/94	2.89	4.51		0,4,4,0	POS 3a	0.165	1.427	>2
F	13/04/94	POS	14.2		4,4,4,0	NEG	0.167	>2	>2

Table 5.15 The Serological Results for the Seroconversion Panel of Samples from Individuals D, E and F.

* EIA

**The reactivity to RIBA antigens is listed in C100, C33c, c22 and NS5 order.

5.7.5.7 Individual G

The first two sequential samples 29/04/94 and 17/11/94 were Abbott EIA negative and absorbances of 0.38 and 0.44 were observed in IMX assay. The PCR status was unknown. These samples did not contain any antibody, which reacted with antigen in either of the NS3 antigen coated plates, or the genotype 3a multiple antigen assay. The third sample taken on 06/03/95 had elevated results in all assays: 4.25 in 2nd generation EIA, 15.8 in IMX: 3,3,4,0 in RIBA: 1.831 with NS3 antigen of genotype 1b; and O.D of 2, in NS3 genotype 3a antigen and BHC129 assays.

5.7.5.8 Individual H

The samples of 12/10/93 and 14/11/94 were not reactive with any of the assays. RIBA test results were not available for individual H. The sample of 27/02/95 was strongly reactive in all the commercial assays and the genotype 3a multiple antigen assay ELISA. This sample was also strongly reactive in the genotype 3a NS3 antigen coated plates, but not reactive in the genotype 1b antigen coated plates.

5.7.5.9 Individual I

The first two samples of 21/09/87 and 13/06/94 were negative by 2nd generation EIA with measured absorbance of 0.46 and 0.44 by IMX. The samples were unreactive with genotype 1b and genotype 3a NS3 antigens and the first sample was unreactive in the genotype 3a multiple antigen assay. The second sample was not available to test with the BHC129 assay. The third sample (24/02/95) was reactive in the 2nd generation EIA (2.86) and the IMX (7.18) assays and RIBA confirmed with 3+ RIBA NS3 and NS4 bands. This sample was reactive with the genotype 3a NS3 antigen (1.422), but not the genotype 1b NS3 assays (0.047), and was also strongly reactive with the genotype 3 multiple antigen assay (Table 5.16).

Individual	Date of Sample	Abbott Generation EIA	Abbott 2 nd Generation IMX*	RIBA 3**	NS3 1b	NS3 Genotype	NS3 3a	Genotype	BHC129 Assay
G	29/09/94	NEG	0.38	NA	0.039		0.03		0.263
G	17/11/94	NEG	0.44	NA	0.019		0.03		0.067
G	06/03/95	4.25	15.8	3,3,4,0	1.831		>2		>2
H	12/10/93	NEG	0.41	NA	0.009		0.04		0.082
H	14/11/94	NEG	0.37	NA	0.025		0.092		0.092
H	27/02/95	2.69	6	4,3,3,0	0.21		>2		>2
I	21/09/87	NEG	0.46	NA	0.021		0.007		0.001
I	13/06/94	NEG	0.44	NA	0.106		0.007		NA
I	24/02/95	2.86	7.18	0,3,3,0	0.047		1.422		>2

Table 5.16 The Serological Results for the Seroconversion Panel of Samples from Individuals G, H and I.

5.7.5.10 Individual J

Individual J was a genotype 3a infected patient who cleared serum RNA in response to interferon treatment (details of INF and treatment regime unknown) and is the opposite of a seroconversion. The date the patient commenced the treatment was also unknown. The 2nd generation EIA reactivity measured was increasing until the sample of 18/10/93 (3.3), which then decreased to 0.66 by the final sample of 10/11/94 (Table 5.17). The 3rd generation measured reactivity also peaked at the 18/10/93 sample and then declined. The IMX reactivity measured fluctuates during the time period. The NS3 genotype 3a antigen reactivity was considerably greater than the NS3 type 1b antigen reactivity for each sample time point, with NS3 genotype 1b antigen reactivity disappearing (sample taken on 02/11/93) before the NS3 genotype 3a antigen reactivity (sample taken on 10/08/94).

5.7.5.11 Control samples

Of the control samples included, all of the samples considered negative by Dr Widdell were unreactive in the experimental assays (Table 5.18). The genotype 1b and 2b mixed infection sample was reactive (>2) in all tests. The genotype 2b sample was detected by the NS3 genotype 3a single antigen assay (>2) and the genotype 3a multiple antigen assay (1.926), but not the NS3 genotype 1b single antigen assay (0.058). Of the 2 genotype 4 samples, one was reactive to the genotype 3a multiple antigen assay (>2), the other was not. Both samples were more reactive in the NS3 genotype 1b antigen assay (1.101) than in the genotype 3a antigen assay (0.174, 0.533). The sample that was identified as a HCV infection in the window period was reactive in the genotype 3a multiple antigen assay, but not reactive in either of the NS3 single antigen plates.

Individual	Date of Sample	Abbott 2 nd Generation EIA	Abbott 2 nd Generation IMX*	Ortho 3 rd Generation EIA	PCR Genotype	/	NS3 Genotype 1b	NS3 Genotype 3a	BHC129 Assay
J	07/10/93	2.64	1.14	0.41	POS 3a		0.222	0.594	0.519
J	11/10/93	3.86	1.86	1.55	NA		0.531	1.432	1.956
J	14/10/93	3.41	1.8	2.23	POS		0.468	1.987	>2
J	18/10/93	3.3	1.51	1.43	NA		0.629	>2	>2
J	25/10/93	1.88	0.96	0.54, 0.34	NA		0.629	>2	>2
J	02/11/93	1.78	0.96	0.87	NA		0.248	>2	>2
J	12/11/93	1.25	1.22	0.69	POS		0.084	1.443	>2
J	24/11/93	1.03	1.5	NA	NEG		0.095	1.878	>2
J	14/12/93	1.78	2.47	NA	NA		0.107	1.629	>2
J	16/02/94	1.25	3.08	NA	NA		0.034	0.773	>2
J	10/08/94	0.8	2.47	NA	NA		0.015	0.193	0.563
J	10/11/94	0.66	2.31	NA	NEG		0.031	0.091	0.152

Table 5.17 The Serological Results for the Seroconversion Panel of Samples from Individual J.

Individual	PCR/ Genotype	NS3 Genotype 1B	NS3 Genotype 3A	BHC129 Assay
Control 1	NEG	0.009	0.016	0
Control 2	NEG	0.005	0.004	0
Control 3	NEG	0.036	0.042	0
Control 4	NEG	0.43	0.058	0
Control 5	POS	0.013	0.003	1.343
Control 6	POS 1B/2B	>2	>2	>2
Control 7	POS 2B	0.058	>2	1.926
Control 8	POS 4	1.101	0.174	>2
Control 9	POS 4	0.553	0.164	0
Control 10	POS 6	>2	>2	>2

Table 5.18 The Serological Results for the Control Samples Provided by Dr Widdell.

The genotype of sample 5 was not provided.

5.7.6 Interpretation

Reactivity to the genotype 3 antigens was stronger than that to the genotype 1 antigens in the majority of the seroconversion panel samples studied. However, the detection of antibody does not appear to be improved by use of the genotype 3a antigen assay.

The RIBA test in early samples from individuals A-F detected core antigen as well as NS3 in the initial response. The lack of sequence variation and the extent of genotype cross-reactive immunity to this region, may explain why these samples were equally well detected in the commercial assays.

One control sample (5) was detected by the genotype 3 antigen screening assay and no other results were available. The information provided about this sample was that it was in the window period of HCV infection. Further discussion with Dr Widdell confirmed that this sample was reactive with 3rd generation screening assays (Ortho EIA 3), but had been included in the panel since it was 2nd generation screening assay negative (Ortho EIA 2).

The control samples of genotype 4 infection reacted better with the antigens of genotype 1b, whereas, the sample of genotype 2b infection reacted better with the genotype 3a antigen. This phenomenon may reflect the extent of similarity of the epitopes between genotype 1 and the genotype 4 infecting virus and genotype 3 and the genotype 2 infecting virus. Also, the genotype 1 antigen may be a better antigen as a result of research and development of the assay. The antibody response to infection persists long after the infection has resolved. This was observed in individual J, who cleared virus in response to interferon therapy. The response was still detectable by Abbott 2nd generation IMX test, a year after the first serum PCR negative result.

The immune response detected by the genotype 3 antigens was stronger than that detected by the genotype 1 assay. This suggests either the response is stronger because of type-specific response or that the genotype 3 assay is more sensitive. If the genotype 3 assay were more sensitive, then the genotype 4 samples would not be better detected by the genotype 1 assay. It is likely that the declining response seen in individual J is similar to the response found in the blood donor populations in Saudi Arabia and Pakistan.

- **The O.D. measured in assays with homologous antigen were higher than heterologous**
- **No instances of earlier detection in seroconversion panels were observed with non genotype 1 antigens.**
- **The response to homologous genotype antigen is detectable for longer after infection has resolved.**

5.8 Samples PCR positive but conventional antibody assay negative or discrepant.

5.8.1 Introduction

Having been unable to find samples which were PCR positive and which contained antibodies which were reactive only with antigen of their infecting genotype in screening donor populations, cryptogenic hepatitis patients or seroconversion panels, the "PCR positive - antibody negative" samples were now examined. These samples, which were negative or discrepant in commercial assays, may contain an antibody response, which was specific to the virus of infection and possibly a non-type 1 genotype, which would be detected with the non-genotype 1 antigen tests.

A number of samples were identified, and sent to the Molecular Virology Laboratory for supplementary testing and analysis, which were found to be PCR positive for HCV, but were discrepant in conventional assay or were without any measurable antibody response. These samples were detected primarily through PCR testing of blood donations and commercial blood product testing and one sample from a liver unit patient who tested negative in commercial assays.

5.8.2 Samples

Mr S Clelland of the SNBTS identified 3 donors from PCR testing of plasma pools from which blood products were to be manufactured (personal communication). These samples were antibody negative in third generation assay when originally tested by SNBTS and when tested by Murex VK48 assay. Of these samples, two were found to be genotype 1 and the third identified as genotype 3 by RFLP of the amplified 5' non-coding region (as described in section 2.1.2.1). Dr V Schottstedt of the DRK Blood Transfusion Service in Hagen, Germany also provided 3 samples. These samples were identified during PCR screening of blood donors. The samples were identified as genotype 1 by RFLP of the 5' non-coding region. A further sample was sent from Dr R Kaiser of the Rheinische Friedrich-Wilhelms University in Bonn, Germany. This serum sample came from a patient who was PCR positive for HCV, but was not positive in commercial serology assays.

5.8.3 Assays

The SNBTS samples and the sample from Bonn were tested in the genotype 1b and genotype 3a NS3 antigen only plates and all the samples were tested in the BHC129 genotype 3a antigen assay.

5.8.4 Results

None of the PCR positive antibody negative samples was positive in any of the antigen assays.

5.8.5 Interpretation

The samples showed no reactivity to antigens of either genotype 1 or genotype 3. The samples from Hagen were sequenced in 5' non-coding and in core regions and showed no abnormalities compared with published sequences. It seems likely that the antibody response in these samples is immature, or absent, rather than specific to antigens only present in the infecting virus.

With these blood donor samples it is unknown when infection took place. This could be a relatively recent occurrence and the individuals could be in the window phase of infection. Another explanation for this finding could be that the individuals may have a genetic, or other underlying, condition, which affects the immune response to these antigens. The sample from the patient who did not seroconvert could, again, be due to a window phase infection, or perhaps, the patient is co- infected with HIV or using immuno-suppressive medication that has caused the immuno-suppression.

- **Use of non genotype one single antigens or multiple antigen assay did not help detect infection in the samples which were PCR positive but commercial assay negative**

Chapter 6

6 Discussion of findings from experimental work

From investigation of single recombinant antigen assays;

- **A genotype specific response is directed at each antigen used in current screening assays.**

From investigation of combined recombinant antigen assays;

- **A genotype specific response is directed at the combination of antigen used in current screening assays.**

Investigation of the impact of type specificity;

- **A five fold reduction in the sensitivity of anti-HCV antibody detection by Ortho 3rd generation assay results in under-detection of individuals with resolved HCV infection.**
- **A 13.5 loss in sensitivity of this assay would result in under-detection of active HCV infection.**

Investigation of detection of infection with non genotype 1 antigens in blood donor populations where other genotypes are predominant;

- **In Saudi Arabian blood donor populations, including individuals of Egyptian origin, genotype-specific response was detected by genotype 4 sequence antigens alone, however, it is likely that it detected a depleting immune response in resolving infection**
- **Genotype-specific anti-HCV antibody response was detected in the blood donor population in Pakistan. However, genotype-specific response to genotype 1b was more frequently observed than response to genotype 3a.**
- **Detection of anti-HCV positive samples was increased by 1.5% when screened by 3rd generation compared to second generation screening assays.**
- **Detection was not improved by screening for surrogate markers of ALT elevation and anti-HBV antibody.**

Investigation of confirmation of infection with non genotype 1 antigens in blood donor populations;

- **Use of NS3 antigens of non-genotype 1 did not allow RIBA “confirmation” of the two true infection cases (PCR positive samples).**
- **Use of the NS3 antigens “confirmed” specific anti-HCV antibody in 5 RIBA unconfirmed cases; these may represent resolved infection.**

Investigation of detection of infection with non genotype 1 antigens in patient populations where other genotypes are predominant;

- **The study of the liver unit patients from Pakistan has identified two problems with screening for HCV by 2nd generation assay. One is that the number of false negatives is high; the other is that there are a number of patients who do not have a detectable antibody response.**
- **The use of genotype 3 antigens in this group would not have improved the detection of HCV in this population.**

Investigation of time scale of detection of infection with non genotype 1 antigens in seroconversion panels of non genotype 1 infection;

- **The O.D. measured in assays with homologous antigen were higher than heterologous**
- **No instances of earlier detection in seroconversion panels were observed with non genotype 1 antigens.**
- **The response to homologous genotype antigen is detectable for longer after infection has resolved.**

Investigation of detection of infection with non genotype 1 antigens in infections where HCV RNA can be detected but anti-HCV antibody can not be detected by conventional assay;

- **Use of non genotype one single antigens or multiple antigen assay did not help detect infection in the samples which were PCR positive but commercial assay negative**

The first aim of this project was to investigate whether the immunogenic regions and epitopes are influenced by the genetic variability of HCV, and to investigate the implications this has for the effectiveness of anti-HCV antibody screening. The results showed type-specific immune responses are directed against each antigen contained in an EIA and demonstrated that the HCV directed antibody response to non-genotype 1 viral infections is under-detected in 3rd generation assays. However, there is no evidence that the lack of detection compromises the safety of blood transfusion as the donors found to be falsely-negative in genotype 1-based serology assays were invariably non-viraemic.

6.1 Investigation of the level of detection of heterologous HCV genotype infection

In a previous study, by Dhaliwal and colleagues (Dhaliwal et al., 1996), it was shown that antibody levels were higher in donors who were actively infected with HCV with detectable viral RNA in plasma. Furthermore, mean antibody reactivity in genotype 1-infected individuals (*ie.* homologous infection), was 4.5-fold greater than in genotype 2 and 3 infection. Using samples from a wider range of individuals, I have been able to confirm these findings, and demonstrated that the combination of non-genotype 1 infection and lack of detectable HCV RNA resulted in substantial under-detection of infection with the current screening assays.

Detection of a type-specific response to genotype 2, 3 and 4 antigens, in seronegative blood donors, suggested that some previously HCV infected donors are undetected with 3rd generation EIA (sections 5.3, 5.4 and 5.5).

6.2 To investigate if the use of heterologous antigens can detect infections undetectable by current serological methods, but detected by molecular biology

Thorough analysis of seronegative PCR positive blood donations, seroconversion panels and sera from liver unit patients provided no evidence that the use of heterologous antigens could aid serological detection of anti-HCV antibodies. There are several possible reasons for this, but the most likely reason is that the immune response was too immature to be detected. In these instances isolation of viral RNA is the only way to detect infection.

6.3 To investigate if use of heterologous HCV genotype antigens can resolve confirmational assay indeterminate samples

In the investigations described, the use of heterologous antigens did not increase the rate of confirmation of PCR positive RIBA indeterminate samples (section 5.5). However, few PCR positive samples were available for analysis. The use of heterologous antigens did increase the rate of detection in PCR negative, anti-HCV antibody indeterminate donors. Detection of reactivity directed against two HCV antigens confirmed the reactivity is not likely to be non-specific or cross-reactive. It is likely that the individuals detected had previously been infected with HCV and spontaneously resolved the infection.

6.4 Investigation if heterologous HCV antigen assays can increase sensitivity of detection in the “window phase”

Through study of both seroconversion panels (section 5.7) and reported serologically negative PCR positive individuals (section 5.8), no evidence was found that use of heterologous antigens would decrease the time to detection of seroconversion.

6.5 The overall aim is to ascertain which combination of antigens best detects infection with all genotypes

From these studies, I concluded that adding antigens of other genotypes does not greatly enhance the sensitivity of screening in a blood donor setting. While some (past, resolved) HCV infections may be undetectable using commercially available type 1-based screening assays, the lack of viraemia in these samples provides no evidence, at present, that these serological "misses" influence the safety of blood transfusion. However, the results do indicate that a proportion of past HCV infections may remain undetected by current diagnostic methods. This work demonstrated that the main cause for concern in the hospitals and blood transfusion services studied was the extent of false negative results generated by the Abbott 2nd Generation EIA. It is likely that this is the true reason for the reported transmission of HCV, despite screening, in these centres.

The most reliable method of screening blood is to test for viral RNA. This, unfortunately, may be too costly for the countries where HCV is most prevalent.

6.6 Nucleotide Amplification Technology

Since July 1999 every blood donation has had to be additionally tested by PCR for HIV and HCV RNA. The testing by PCR is estimated to reduce the time of the window period, that is the time between infection and the individual having sufficient markers of infection to detect, to between 10-29 days. In the year following the introduction of testing SNBTS have identified three infective samples that were serologically negative. This means that prior to PCR screening 1:840 000 donations was infectious.

6.7 How has this work advanced the current understanding of HCV serology?

The main finding is that the magnitude of the antibody response to infection is not influenced by the genotype of infection. The lower serological reactivity detected in conventional serology assays results from the presence of type-specific epitopes present in the recombinant antigens.

Although the use of heterologous antigens does not increase the rate of detection of new infections or resolve indeterminate results, it does allow the detection of many previous infections. This may be beneficial in epidemiological studies or in estimates of rates of HCV resolution in populations.

The introduction of heterologous antigens in current screening assays would reduce assay specificity, and therefore increase the number of false positive results that would have to be resolved by supplementary or confirmatory antibody tests and PCR. However, this work demonstrated that a large number of “false” negative results are still generated in areas of high prevalence.

The majority of the individuals studied in this thesis were healthy blood donors, and it is possible that greater improvement in the detection rate of non-type 1 genotypes would be achieved in individuals with impaired immune responses to infection and incomplete antibody repertoires. The effect of concurrent disease, coinfection, genetic factors and immunosuppressive drugs are each likely to diminish the detectable immune response. In these cases the use of heterologous antigens may be useful and should be further investigated.

References

- Afonso, A. M. R., Jiang, J. J., Penin, F., Tareau, C., Samuel, D., Petit, M. A., Bismuth, H., Dussaix, E., and Feray, C. 1999. Nonrandom distribution of hepatitis C virus quasispecies in plasma and peripheral blood mononuclear cell subsets. *J Virol* 73[11], 9213-9221.
- Agnello, V., Abel, G., Elfahal, M., Knight, G. B., and Zhang, Q. X. 1999. Hepatitis C virus and other Flaviviridae viruses enter cells via low density lipoprotein receptor. *Proc Nat Acad Sci Usa* 96[22], 12766-12771
- Akatsuka T, Donets M, Scaglione L, Ching WM, Shih JW, Di Bisceglie AM, Feinstone SM. 1993 .B-cell epitopes on the hepatitis C virus nucleocapsid protein determined by human monospecific antibodies. *Hepatology*. Sep;18(3):503-10
- Al, R. H., Xie, Y. P., Wang, Y. H., and Hagedorn, C. 1998. H. Expression of recombinant hepatitis C virus non-structural protein 5B in *Escherichia coli*. *Virus Res*. 53[2], 141-149.
- Alter HJ, Seeff LB. 2000. Recovery, persistence, and sequelae in hepatitis C virus infection: a perspective on long-term outcome. *Semin Liver Dis*. 2000;20(1):17-35.
- Ansaldi F, Torre F, Bruzzzone BM, Picciotto A, Crovari P, Icardi G. Evaluation of a new hepatitis C virus sequencing assay as a routine method for genotyping. *J Med Virol*. 2001 Jan;63(1):17-21.
- Barrett S, Ryan E &. Crowe J, Association of the HLA-DRB1*01 allele with spontaneous viral clearance in an Irish cohort infected with hepatitis C virus via contaminated anti-D immunoglobulin, *J. Hepatol.*, 1999, Jun 30(6), 979-83.
- Barrera, J. M., B. Francis , G. Ercilla, M. Nelles, D. Achord, J. Darner, and S. R. Lee. 1995. Improved detection of anti-HCV in post-transfusion hepatitis by a third-generation ELISA. *Vox Sang*. 68:15-18.
- Bartenschlager, R., L. Ahlbornlaake, J. Mous, and H. Jacobsen. 1994. Kinetic and structural analyses of hepatitis C virus polyprotein processing. *J. Virol*. 68:5045-5055.
- Bartosch B, Vitelli A, Granier C, Goujon C, Dubuisson J, Pascale S, Scarselli E, Cortese R, Nicosia A, Cosset FL. Cell entry of hepatitis C virus requires a set of co-receptors that include the CD81 tetraspanin and the SR-B1 scavenger receptor *J Biol Chem*. 2003 Aug 11
- Behrens, S. E., Tomei, L., and DeFrancesco, R. 1996. Identification and properties of the RNA-dependent RNA polymerase of hepatitis C virus. *EMBO J*. 15[1], 12-22.

- Bhattacharjee, V., L. E. Prescott, I. Pike, B. Rodgers, H. Bell, A. R. Elzayadi, M. C. Kew, J. Conradie, C. K. Lin, H. Marsden, A. A. Saeed, D. Parker, P. L. Yap, and P. Simmonds. 1995. Use of NS-4 peptides to identify type-specific antibody to hepatitis C virus genotypes 1, 2, 3, 4, 5 and 6. *J. Gen. Virol.* 76:1737-1748.
- Blight, K., R. R. Lesniewski, J. T. LaBrooy, and E. J. Gowans. 1994. Detection and distribution of hepatitis C-specific antigens in naturally infected liver. *Hepatology* 20:553-557
- Bonkovsky HL, Poh-Fitzpatrick M, Pimstone N, Obando J, Di Bisceglie A, Tattie C, Tortorelli K, LeClair P, Mercurio MG, Lambrecht RW. 1998 Porphyria cutanea tarda, hepatitis C, and HFE gene mutations in North America. *Hepatology.* 1998 Jun;27(6):1661-9.
- Borowski, P., Heiland, M., Oehlmann, K., Becker, B., Kornetzky, L., Feucht, H., and Laufs, R. 1996. Non-structural protein 3 of hepatitis C virus inhibits phosphorylation mediated by cAMP-dependent protein kinase. *Eur.J.Biochem.* 237[3], 611-618.
- Both, G. W., M. J. Sleight, N. J. Cox, and A. P. Kendal. 1983. Antigenic drift in influenza virus H3 hemagglutinin from 1968 to 1980: multiple evolutionary pathways and sequential amino acid changes at key antigenic sites. *J. Virol.* 48:52-60.
- Bouffard, P., P. H. Hayashi, R. Acevedo, N. Levy, and J. B. Zeldis. 1992. Hepatitis C virus is detected in a monocyte macrophage subpopulation of peripheral blood mononuclear cells of infected patients. *J. Infect. Dis.* 166:1276-1280.
- Bouvier-Alias M, Patel K, Dahari H, Beaucourt S, Larderie P, Blatt L, Hezode C, Picchio G, Dhumeaux D, Neumann AU, McHutchison JG, Pawlotsky JM. Clinical utility of total HCV core antigen quantification: a new indirect marker of HCV replication. *Hepatology.* 2002 Jul;36(1):211-8
- Bradley, D., K. McCaustland, K. Krawczynski, J. Spelbring, C. Humphrey, and E. H. Cook. 1991. Hepatitis C virus: buoyant density of the factor VIII-derived isolate in sucrose. *J. Med. Virol.* 34:206-208.
- Bukh, J., R. H. Purcell, and R. H. Miller. 1992. Sequence analysis of the 5' noncoding region of hepatitis C virus. *Proc. Natl. Acad. Sci. USA* 89:4942-4946.
- Carrick, R. J., G. G. Schlauder, D. A. Peterson, and I. K. Mushahwar. 1992. Examination of the buoyant density of hepatitis-c virus by the polymerase chain reaction. *J. Virol. Methods* 39:279-290.
- Castillo, I., J. Bartolome, J. A. Quiroga, and V. Carreno. 1992. Comparison of several PCR procedures for detection of serum HCV- RNA using different regions of the HCV genome. *J. Virol. Methods* 38:71-79.

- Capelli C, Prati D, Bosoni P, Zanuso F, Pappalettera M, Mozzi F, De Mattei C, Zanella A, Sirchia G. Sexual transmission of hepatitis C virus to a repeat blood donor. *Transfusion*. 1997 Apr;37(4):436-40.
- Chan, S. W., P. Simmonds, F. McOmish, P. L. Yap, R. Mitchell, B. Dow, and E. Follett. 1991. Serological responses to infection with three different types of hepatitis C virus. *Lancet* 338:1391.
- Chayama, K., A. Tsubota, Y. Arase, S. Saitoh, I. Koida, K. Ikeda, T. Matsumoto, M. Kobayashi, S. Iwasaki, S. Koyama, T. Morinaga, and H. Kumada. 1993. Genotypic subtyping of hepatitis C virus. *J. Gastroenterol. Hepatol.* 8:150-156.
- Chien DY, Choo QL, Tabrizi A, Kuo C, McFarland J, Berger K, Lee C, Shuster JR, Nguyen T, Moyer DL, et al. 1992 Diagnosis of hepatitis C virus (HCV) infection using an immunodominant chimeric polyprotein to capture circulating antibodies: reevaluation of the role of HCV in liver disease. *Proc Natl Acad Sci U S A*. Nov 1;89(21):10011-5
- Choo, Q. L., G. Kuo, A. J. Weiner, L. R. Overby, D. W. Bradley, and M. Houghton. 1989. Isolation of a cDNA derived from a blood-borne non-A, non-B hepatitis genome. *Science* 244:359-362.
- Choo, Q. L., K. H. Richman, J. H. Han, K. Berger, C. Lee, C. Dong, C. Gallegos, D. Coit, R. Medina Selby, P. J. Barr, A. J. Weiner, D. W. Bradley, G. Kuo, and M. Houghton. 1991. Genetic organization and diversity of the hepatitis C virus. *Proc. Natl. Acad. Sci. USA* 88:2451-2455.
- Choo QL, Kuo G, Ralston R, Weiner A, Chien D, Van Nest G, Han J, Berger K, Thudium K, Kuo C, et al. 1994. Vaccination of chimpanzees against infection by the hepatitis C virus. *Proc Natl Acad Sci U S A*. 1994 Feb 15;91(4):1294-8.
- Choo, S. H., H. S. So, J. M. Cho, and W. S. Ryu. 1995. Association of hepatitis C virus particles with immunoglobulin: a mechanism for persistent infection. *J. Gen. Virol.* 76:2337-2341.
- Claeys, H., A. Volckaerts, W. Mertens, Z. Liang, P. Fiten, and G. Opdenakker. 1995. Localization and reactivity of an immunodominant domain in the NS3 region of hepatitis C virus. *J. Med. Virol.* 45:273-281.
- Conry-Cantilena C, VanRaden M, Gobble J, Melpolder J, Shakil AO, Viladomiu L, Cheung L, DiBisceglie A, Hoofnagle J, Shih JW, et al. Routes of infection, viremia, and liver disease in blood donors found to have hepatitis C virus infection. *N Engl J Med*. 1996 Jun 27;334(26):1691-6
- Cooper S, Erickson AL, Adams EJ, Kansopon J, Weiner AJ, Chien DY, Houghton M, Parham P, Walker CM. 1999 Analysis of a successful immune response against hepatitis C virus. *Immunity*. Apr;10(4):439-49.

Davidson, F., P. Simmonds, J. C. Ferguson, L. M. Jarvis, B. C. Dow, E. A. C. Follett, C. R. G. Seed, T. Krusius, C. Lin, G. A. Medgyesi, H. Kiyokawa, G. Olim, G. Duraisamy, T. Cuypers, A. A. Saeed, D. Teo, J. Conradie, M. C. Kew, M. Lin, C. Nuchaprayoon, O. K. Ndimbie, and P. L. Yap. 1995. Survey of major genotypes and subtypes of hepatitis C virus using RFLP of sequences amplified from the 5' non-coding region. *J. Gen. Virol.* 76:1197-1204.

Dhaliwal, S. K., Prescott, L. E., Dow, B. C., Davidson, F., Brown, H., Yap, P. L., Follett, E. A. C., and Simmonds, P. 1996. Influence of viraemia and genotype upon serological reactivity in screening assays for antibody to hepatitis C virus. *J. Med. Virol.* 48[2], 184-190.

Diepolder HM, Zachoval R, Hoffmann RM, Jung MC, Gerlach T, Pape GR. 1996 The role of hepatitis C virus specific CD4+ T lymphocytes in acute and chronic hepatitis C. *J Mol Med.* Oct;74(10):583-8

Dixit, V., S. Quan, P. Martin, D. Larson, M. Brezina, R. Dinello, K. Sra, J. Y. N. Lau, D. Chien, J. Kolberg, A. Tagger, G. Davis, A. Polito, and G. Gitnick. 1995. Evaluation of a novel serotyping system for hepatitis C virus: strong correlation with standard genotyping methodologies. *J. Clin. Microbiol.* 33:2978-2983.

Dow, B. C., Munro, H., Buchanan, I., Follett, E. A. C., Davidson, F., Yap, P. L., and Simmonds, P. 1996. Third-generation recombinant immunoblot assay: Comparison of reactivities according to hepatitis C virus genotype. *Transfusion* 36[6], 547-551.

Eckart, M. R., M. Selby, F. Masiarz, C. Lee, K. Berger, K. Crawford, C. Kuo, G. Kuo, M. Houghton, and Q. L. Choo. 1993. The hepatitis C virus encodes a serine protease involved in processing of the putative nonstructural proteins from the viral polyprotein precursor. *Biochem. Biophys. Res. Commun.* 192:399-406.

Elghouzzi MH, Bouchardeau F, Pillonel J, Boiret E, Tirtaine C, Barlet V, Moncharmont P, Maisonneuve P, du Puy-Montbrun MC, Lyon-Caen D, Courouge AM. Hepatitis C virus: routes of infection and genotypes in a cohort of anti-HCV-positive French blood donors. *Vox Sang.* 2000;79(3):138-44.

Failla, C., L. Tomei, and R. DeFrancesco. 1994. Both NS3 and NS4a are required for proteolytic processing of hepatitis C virus nonstructural proteins. *J. Virol.* 68:3753-3760.

Fang SH, Hwang LH, Chen DS, Chiang BL Ribavirin enhancement of hepatitis C virus core antigen-specific type 1 T-helper cell response correlates with the increased IL-12 level *J Hepatol* 2000 Nov;33(5):791-8

Farci P, Alter HJ, Wong D, Miller RH, Shih JW, Jett B, Purcell RH. 1991 A long-term study of hepatitis C virus replication in non-A, non-B hepatitis. *N Engl J Med.* 1991 Jul 11;325(2):98-104

Farci P, Shimoda A, Wong D, Cabezon T, De Gioannis D, Strazzer A, Shimizu Y, Shapiro M, Alter HJ, Purcell RH. 1996. Prevention of hepatitis C virus infection in chimpanzees by hyperimmune serum against the hypervariable region 1 of the envelope 2 protein. *Proc Natl Acad Sci U S A*. 1996 Dec 24;93(26):15394-9.

Felsenstein, J. 1988. Phylogenies from molecular sequences: inferences and reliability. *Ann. Rev. Genet.* 22:521-565.

Fong, T. L., M. Shindo, S. M. Feinstone, J. H. Hoofnagle, and A. M. Di Bisceglie. 1991. Detection of replicative intermediates of hepatitis C viral RNA in liver and serum of patients with chronic hepatitis C. *J. Clin. Invest.* 88:1058-1060.

Foy E, Li K, Wang C, Sumpter R Jr, Ikeda M, Lemon SM, Gale M Jr. Regulation of interferon regulatory factor-3 by the hepatitis C virus serine protease. *Science*, April 17, 2003.

Gale, M. J., Korth, M. J., Tang, N. M., Tan, S. L., Hopkins, D. A., Dever, T. E., Polyak, S. J., Gretch, D. R., and Katze, M. G. 1997. Evidence that hepatitis C virus resistance to interferon is mediated through repression of the PKR protein kinase by the nonstructural 5A protein. *Virology* 230[2], 217-227.

Gale M Jr, Blakely CM, Kwieciszewski B, Tan SL, Dossett M, Tang NM, Korth MJ, Polyak SJ, Gretch DR, Katze MG. 1998 Control of PKR protein kinase by hepatitis C virus nonstructural 5A protein: molecular mechanisms of kinase regulation. *Mol Cell Biol.* Sep;18(9):5208-18.

Garson, J. A., R. S. Tedder, M. Briggs, P. Tuke, J. A. Glazebrook, A. Trute, D. Parker, J. A. Barbara, M. Contreras, and S. Aloysius. 1990. Detection of hepatitis C viral sequences in blood donations by "nested" polymerase chain reaction and prediction of infectivity. *Lancet* 335:1419-1422.

Gonzalezperalta, R. P., She, J. Y., Davis, G. L., Ohno, T., Mizokami, M., and Lau, J. Y. N. 1996. Clinical implications of viral quasispecies heterogeneity in chronic hepatitis C. *J. Med. Virol.* 49[3], 242-247.

Grakoui, A., D. W. McCourt, C. Wychowski, S. M. Feinstone, and C. M. Rice. 1993. A second hepatitis C virus-encoded proteinase. *Proc. Natl. Acad. Sci. USA* 90:10583-10587.

Grakoui, A., D. W. McCourt, C. Wychowski, S. M. Feinstone, and C. M. Rice. 1993. Characterization of the hepatitis C virus-encoded serine proteinase - determination of proteinase-dependent polyprotein cleavage sites. *J. Virol.* 67:2832-2843.

Gretch, D. R., C. Delarosa, R. L. Carithers, R. A. Willson, B. Williams, and L. Corey. 1995. Assessment of hepatitis C viremia using molecular amplification technologies: correlations and clinical implications. *Ann. Intern. Med.* 123:321-329.

- Gumber SC, Chopra S. 1995 Hepatitis C: a multifaceted disease. Review of extrahepatic manifestations. *Ann Intern Med.* 1995 Oct 15;123(8):615-20.
- Guadagnino V, Stroffolini T, Rapicetta M, Costantino A, Kondili LA, Menniti-Ippolito F, Caroleo B, Costa C, Griffo G, Loiacono L, Pisani V, Foca A, Piazza M. Prevalence, risk factors, and genotype distribution of hepatitis C virus infection in the general population: a community-based survey in southern Italy. *Hepatology.* 1997 Oct;26(4):1006-11.
- Gwack, Y., Kim, D. W., Han, J. H., and Choe, J. 1996. Characterization of RNA binding activity and RNA helicase activity of the hepatitis C virus NS3 protein. *Biochemical and Biophysical Research Communications* 225[2], 654-659.
- Haddad J, Deny P, Munz-Gotheil C, Ambrosini JC, Trinchet JC, Pateron D, Mal F, Callard P, Beaugrand M 1992 Lymphocytic sialadenitis of Sjogren's syndrome associated with chronic hepatitis C virus liver disease. *Lancet.* 1992 Feb 8;339(8789):321-3.
- Hayashi, J., Y. Kishihara, K. Yamaji, N. Furusyo, T. Yamamoto, Y. Pae, Y. Etoh, H. Ikematsu, and S. Kashiwagi. 1997. Hepatitis C viral quasispecies and liver damage in patients with chronic hepatitis C virus infection. *Hepatology* 25:697-701.
- Hausfater P, Cacoub P, Sterkers Y, Thibault V, Amoura Z, Nguyen L, Ghillani P, Leblond V, Piette JC. 2001 Hepatitis C virus infection and lymphoproliferative diseases: prospective study on 1,576 patients in France. *Am J Hematol.* 2001 Jul;67(3):168-71.
- Heckmann JG, Kayser C, Heuss D, Manger B, Blum HE, Neundorfer B. 1999 Neurological manifestations of chronic hepatitis C. *J Neurol.* 1999 Jun;246(6):486-91.
- Hijikata, M., H. Mizushima, Y. Tanji, Y. Komoda, Y. Hirowatari, T. Akagi, N. Kato, K. Kimura, and K. Shimotohno. 1993. Proteolytic processing and membrane association of putative nonstructural proteins of hepatitis C virus. *Proc. Natl. Acad. Sci. USA* 90:10773-10777.
- Hijikata, M., Y. K. Shimizu, H. Kato, A. Iwamoto, J. W. Shih, H. J. Alter, R. H. Purcell, and H. Yoshikura. 1993. Equilibrium centrifugation studies of hepatitis C virus - evidence for circulating immune complexes. *J. Virol.* 67:1953-1958.
- Hirowatari, Y., M. Hijikata, Y. Tanji, H. Nyunoya, H. Mizushima, K. Kimura, T. Tanaka, N. Kato, and K. Shimotohno. 1993. Two proteinase activities in HCV polypeptide expressed in insect cells using baculovirus vector. *Arch. Virol.* 133 :349-356.
- Holland, J., Spindler K., Horodyski F., Grabau E., Nichol S., and VandePol. S. 1982. Rapid evolution of RNA genomes. *Science* 215:1577-1585.
- Holland, J. De La Torre C, and. Steinhauer D. A. 1992. RNA virus populations as quasispecies. *Curr. Top. Microbiol. Immunol.* 176:1-20.

Hultgren, C., D. R. Milich, O. Weiland, and M. Sallberg. 1998. The antiviral compound ribavirin modulates the T helper (Th) 1/Th2 subset balance in hepatitis B and C virus-specific immune responses. *J. Gen. Virol.* 79 (Pt 10):2381-2391.

Ide, Y., Tanimoto, A., Sasaguri, Y., and Padmanabhan, R. 1997 Hepatitis C virus NS5A protein is phosphorylated in vitro by a stably bound protein kinase from HeLa cells and by cAMP-dependent protein kinase A- α catalytic subunit. *Gene* 201[1-2], 151-158..

Ikeda, S., M. Uchida, S. Fukumoto, and Y. Shimada. 1991. [Prevalence of HCV antibodies from mass survey in Shimane Prefecture]. *Nippon. Rinsho.* 49:378-382.

Ishii K, Rosa D, Watanabe Y, Katayama T, Harada H, Wyatt C, Kiyosawa K, Aizaki H, Matsuura Y, Houghton M, Abrignani S, Miyamura T 1998 High titers of antibodies inhibiting the binding of envelope to human cells correlate with natural resolution of chronic hepatitis C *Hepatology.* Oct;28(4):1117-20

Jarvis, L. M., H. G. Watson, F. McOmish, J. F. Peutherer, C. A. Ludlam, and P. Simmonds. 1994. Frequent reinfection and reactivation of hepatitis C virus genotypes in multitransfused hemophiliacs. *J. Infect. Dis.* 170:1018-1022.

Jenkins, P. J., Cromie, S. L., Bowden, D. S., Finch, C. F., and Dudley, F. J. 1996. Chronic hepatitis C and interferon alfa therapy: Predictors of long term response. *Med. J. Aust.* 164[3], 150-152.

Kaito, M., S. Watanabe, K. Tsukiyamakohara, K. Yamaguchi, Y. Kobayashi, M. Konishi, M. Yokoi, S. Ishida, S. Suzuki, and M. Kohara. 1994. Hepatitis C virus particle detected by immunoelectron microscopic study. *J. Gen. Virol.* 75:1755-1760.

Kanto, T., N. Hayashi, T. Takehara, H. Hagiwara, E. Mita, M. Naito, A. Kasahara, H. Fusamoto, and T. Kamada. 1994. Buoyant density of hepatitis C virus recovered from infected hosts - two different features in sucrose equilibrium density- gradient centrifugation related to degree of liver inflammation. *Hepatology* 19:296-302.

Kanto, T., N. Hayashi, T. Takehara, H. Hagiwara, E. Mita, M. Naito, A. Kasahara, H. Fusamoto, and T. Kamada. 1995. Density analysis of hepatitis C virus particle population in the circulation of infected hosts: implications for virus neutralization or persistence. *J. Hepatol.* 22:440-448.

Kato, N., Ikeda, M., Sugiyama, K., Mizutani, T., Tanaka, T., and Shimotohno, K. 1998. Hepatitis C virus population dynamics in human lymphocytes and hepatocytes infected in vitro. *Journal of General Virology* 79, 1859-1869.

Kenny-Walsh E. Clinical outcomes after hepatitis C infection from contaminated anti-D immune globulin. Irish Hepatology Research Group. *N Engl J Med.* 1999 Apr 22;340(16):1228-33.

Koff RS, Dienstag JL. 1995 Extrahepatic manifestations of hepatitis C and the association with alcoholic liver disease. *Semin Liver Dis.* 1995 Feb;15(1):101-9.

- Kolykhalov, A. A., Feinstone, S. M., and Rice, C. M. 1996 Identification of a highly conserved sequence element at the 3' terminus of hepatitis C virus genome RNA. *Journal of Virology* 70[6], 3363-3371.
- Koziel MJ, Wong DK, Dudley D, Houghton M, Walker BD. 1997 Hepatitis C virus-specific cytolytic T lymphocyte and T helper cell responses in seronegative persons. *J Infect Dis.* 1997 Oct;176(4):859-66.
- Kudo, T., Yanase, Y., Ohshiro, M., Yamamoto, M., Morita, M., Shibata, M., and Morishima, T. 1997. Analysis of mother-to-infant transmission of hepatitis C virus: Quasispecies nature and buoyant densities of maternal virus populations. *Journal of Medical Virology* 51[3], 225-230.
- Lanford, R. E., D. Chavez, F. Vonchisari, and C. Sureau. 1995. Lack of detection of negative-strand hepatitis C virus RNA in peripheral blood mononuclear cells and other extrahepatic tissues by the highly strand-specific reverse transcriptase PCR. *J. Virol.* 69:8079-8083.
- Large MK, Kittlesen DJ, Hahn YS 1999 Suppression of host immune response by the core protein of hepatitis C virus: possible implications for hepatitis C virus persistence. *J Immunol.* Jan 15;162(2):931-8.
- Lau, J. Y. N., Davis, G. L., Prescott, L. E., Maertens, G., Lindsay, K. L., Qian, K. P., Mizokami, M., Simmonds, P., Perrillo, R. P., Schiff, E. R., Bodenheimer, H. C., Balart, L. A., Regenstien, F., Dienstag, J. L., Katkov, W. N., Tamburro, C. H., Goff, J. S., Everson, G. T., Goodman, Z., and Albrecht, J. 1996 Distribution of hepatitis C virus genotypes determined by line probe assay in patients with chronic hepatitis C seen at tertiary referral centers in the United States. *Ann.Intern.Med.* 124[10], 868.
- Lau, J. Y. N., M. Mizokami, J. A. Kolberg, G. L. Davis, L. E. Prescott, T. Ohno, R. P. Perrillo, K. L. Lindsay, R. G. Gish, K. P. Qian, M. Kohara, P. Simmonds, and M. S. Urdea. 1995. Application of six hepatitis C virus genotyping systems to sera from chronic hepatitis C patients in the United States. *J. Infect. Dis.* 171:281-289.
- Lamonaca V, Missale G, Urbani S, Pilli M, Boni C, Mori C, Sette A, Massari M, Southwood S, Bertoni R, Valli A, Fiaccadori F, Ferrari C. 1999 Conserved hepatitis C virus sequences are highly immunogenic for CD4(+) T cells: implications for vaccine development. *Hepatology.* Oct;30(4):1088-98.
- Lawal, Z., Petrik, J., Wong, V. S., Alexander, G. J. M., and Allain, J. P. 1997. Hepatitis C virus genomic variability in untreated and immunosuppressed patients. *Virology* 228[1], 107-111.
- Lelie, P. N., H. T. Cuypers, H. W. Reesink, C. L. van der Poel, I. Winkel, E. Bakker, P. J. Exel Oehlers, D. Vallari, J. P. Allain, and L. Mimms. 1992. Patterns of serological markers in transfusion-transmitted hepatitis C virus infection using second generation HCV assays. *J. Med. Virol.* 37:203-209.
- Levy, S., S. C. Todd, and H. T. Maecker. 1998. CD81 (TAPA-1): a molecule involved in signal transduction and cell adhesion in the immune system. *Annu. Rev. Immunol.* 16:89-109.

- Liang TJ, Rehermann B, Seeff LB, Hoofnagle JH. 2000 Pathogenesis, natural history, treatment, and prevention of hepatitis C *Ann Intern Med.* 2000 Feb 15;132(4):296-305.
- Li, X., L. J. Jeffers, L. Shao, K. R. Reddy, M. De Medina, J. Scheffell, B. Moore, and E. R. Schiff . 1995. Identification of hepatitis C virus by immunoelectron microscopy. *J. Viral Hepat.* 2:227-234.
- Linnen, J., J. Wages, Z. Zhang-Keck, K. E. Fry, K. Z. Krawczynski, H. Alter, E. Koonin, M. Gallagher, M. Alter, and et al. 1996. Molecular cloning and disease association of hepatitis G virus: a transfusion-transmissible agent. *Science* 271:505-508.
- Lohmann, V., F. Korner, U. Herian, and R. Bartenschlager. 1997. Biochemical properties of hepatitis C virus NS5B RNA- dependent RNA polymerase and identification of amino acid sequence motifs essential for enzymatic activity. *J. Virol.* 71:8416-8428.
- Lohmann, V., Korner, F., Koch, J. O., Herian, U., Theilmann, L., and Bartenschlager, R. 1999. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science* 285[5424], 110-113.
- Machida, A., H. Ohnuma, F. Tsuda, E. Munekata, T. Tanaka, Y. Akahane, H. Okamoto, and S. Mishiro. 1992. Two distinct subtypes of hepatitis C virus defined by antibodies directed to the putative core protein. *Hepatology* 16:886-891.
- Maggi, F., Fornai, C., Vatteroni, M. L., Giorgi, M., Morrica, A., Pistello, M., Cammarota, G., Marchi, S., Ciccorossi, P., Bionda, A., and Bendinelli, M. 1997. Differences in hepatitis C virus quasispecies composition between liver, peripheral blood mononuclear cells and plasma. *Journal of General Virology* 78, 1521-1525.
- Manns, M. P., J. G. McHutchison, S. C. Gordon, V. K. Rustgi, M. Shiffman, R. Reindollar, Z. D. Goodman, K. Koury, M. Ling, and J. K. Albrecht. 2001. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* 358:958-965.
- McAllister J, Casino C, Davidson F, Power J, Lawlor E, Yap PL, Simmonds P, Smith DB. 1998 Long-term evolution of the hypervariable region of hepatitis C virus in a common-source-infected cohort. *J Virol.* Jun;72(6):4893-905.
- McHutchison, J. G. and T. Poynard. 1999. Combination therapy with interferon plus ribavirin for the initial treatment of chronic hepatitis C. *Semin. Liver Dis.* 19 Suppl 1:57-65.
- McOmish, F., S.-W. Chan, B. C. Dow, J. Gillon, W. D. Frame, R. J. Crawford , P. L. Yap, E. A. C. Follett, and P. Simmonds. 1993. Detection of three types of hepatitis C virus in blood donors: Investigation of type-specific differences in serological reactivity and rate of alanine aminotransferase abnormalities. *Transfusion* 33:7-13.

McOmish, F., P. L. Yap, B. C. Dow, E. A. C. Follett, C. Seed, A. J. Keller, T. J. Cobain, T. Krusius, E. Kolho, R. Naukkarinen, C. Lin, C. Lai, S. Leong, G. A. Medgyesi, M. Hejjas, H. Kiyokawa, K. Fukada, T. Cuypers, A. A. Saeed, A. M. Alrasheed, M. Lin, and P. Simmonds. 1994. Geographical distribution of hepatitis C virus genotypes in blood donors - an international collaborative survey . *J. Clin. Microbiol.* 32:884-892.

Mellor, J., E. C. Holmes, L. M. Jarvis, P. L. Yap, P. Simmonds, and International Collaborators. 1995. Investigation of the pattern of hepatitis C virus sequence diversity in different geographical regions: implications for virus classification. *J. Gen. Virol.* 76:2493-2507.

Memon MI, Memon MA. Hepatitis C: an epidemiological review *J Viral Hepat.* 2002 Mar;9(2):84-100

Misiani R, Bellavita P, Fenili D, Vicari O, Marchesi D, Sironi PL, Zilio P, et al. Interferon alfa -2a therapy in cryoglobulinemia associated with hepatitis C virus. *N Engl J Med* 1994;330:751-756

Mizokami, M., J. Y. N. Lau , K. Suzuki, T. Nakano, and T. Gojobori. 1994. Differential sensitivity of hepatitis C virus quasispecies to interferon- α therapy. *J. Hepatol.* 21:884-886.

Monazahian, M., Bohme, I., Bonk, S., Koch, A., Scholz, C., Grethe, S., and Thomssen, R. 1999. Low density lipoprotein receptor as a candidate receptor for hepatitis C virus. *J Med Virol* 57[3], 223-229.

Mondelli, M. U., A. Cerino , F. Bono, A. Cividini, A. Maccabruni, M. Arico, A. Malfitano, G. Barbarini, V. Piazza, L. Minoli, and E. Silini. 1994. Hepatitis C virus (HCV) core serotypes in chronic HCV infection. *J. Clin. Microbiol.* 32:2523-2527.

Moore, J. P., J. A. McKeating, W. A. Norton, and Q. J. Sattentau. 1991. Direct measurement of soluble CD4 binding to human immunodeficiency virus type 1 virions: gp120 dissociation and its implications for virus- cell binding and fusion reactions and their neutralization by soluble CD4. *J. Virol.* 65:1133-1140.

Muller, H. M., E. Pfaff, T. Goeser, B. Kallinowski , C. Solbach, and L. Theilmann. 1993. Peripheral blood leukocytes serve as a possible extrahepatic site for hepatitis C virus replication. *J. Gen. Virol.* 74:669-676.

Murphy, D., B. Willems, and G. Delage. 1994. Use of the 5' non-coding region for genotyping hepatitis C virus. *J. Infect. Dis.* 169:473-475.

Nafeh MA, Medhat A, Shehata M, Mikhail NN, Swiffee Y, Abdel-Hamid M, Watts S, Fix AD, Strickland GT, Anwar W, Sallam I. 2000 Hepatitis C in a community in Upper Egypt: I. Cross-sectional survey *Am J Trop Med Hyg.* 2000 Nov-Dec;63(5-6):236-41.

Neumann, A. U., Lam, N. P., Dahari, H., Gretch, D. R., Wiley, T. E., Layden, T. J., and Perelson, A. S. 1998. Hepatitis C viral dynamics in vivo and the antiviral efficacy of interferon- α therapy. *Science* 282[5386], 103-107.

- Nouri-Aria KT, Sallie R, Mizokami M, Portmann BC, Williams R. 1995. Intrahepatic expression of hepatitis C virus antigens in chronic liver disease. *J Pathol.* Jan;175(1):77-83
- Okamoto, H., M. Kojima, S.-I. Okada, H. Yoshizawa, H. Iizuka, T. Tanaka, E. E. Muchmore, Y. Ito, and S. Mishiro. 1992. Genetic drift of hepatitis C virus during an 8.2 year infection in a chimpanzee: variability and stability. *Virology* 190:894-899.
- Okamoto, H., S. Okada, Y. Sugiyama, K. Kurai, H. Iizuka, A. Machida, Y. Miyakawa, and M. Mayumi. 1991. Nucleotide sequence of the genomic RNA of hepatitis C virus isolated from a human carrier: comparison with reported isolates for conserved and divergent regions. *J. Gen. Virol.* 72:2697-2704.
- Okamoto, H., S. Okada, Y. Sugiyama, S. Yotsumoto, T. Tanaka, H. Yoshizawa, F. Tsuda, Y. Miyakawa, and M. Mayumi. 1990. The 5'-terminal sequence of the hepatitis C virus genome. *Jpn. J. Exp. Med.* 60:167-177.
- Okamoto, H., Y. Sugiyama, S. Okada, K. Kurai, Y. Akahane, Y. Sugai, T. Tanaka, K. Sato, F. Tsuda, Y. Miyakawa, and M. Mayumi. 1992. Typing hepatitis C virus by polymerase chain reaction with type-specific primers: application to clinical surveys and tracing infectious sources. *J. Gen. Virol.* 73:673-679.
- Pavlovic D, Neville DC, Argaud O, Blumberg B, Dwek RA, Fischer WB, Zitzmann N. The hepatitis C virus p7 protein forms an ion channel that is inhibited by long-alkyl-chain iminosugar derivatives. *Proc Natl Acad Sci U S A.* 2003 May 13;100(10):6104-8. Epub 2003 Apr 28
- Pawlotsky, J. M., M. Ben Yahia, C. Andre, M. C. Voisin, L. Intrator, F. Roudot-Thoraval, L. Deforges, C. Duvoux, E. S. Zafrani, J. Duval, and . 1994. Immunological disorders in C virus chronic active hepatitis: a prospective case-control study. *Hepatology* 19:841-848.
- Pawlotsky, J. M., Pellerin, M., Bouvier, M., Roudotthoraval, F., Germanidis, G., Bastie, A., Darthuy, F., Remire, J., Soussy, C. J., and Dhumeaux, D. 1998. Genetic complexity of the hypervariable region 1 (HVR1) of hepatitis C virus (HCV): Influence on the characteristics of the infection and responses to interferon alfa therapy in patients with chronic hepatitis C. *Journal of Medical Virology* 54[4], 256-264.
- Petracca, R., F. Falugi, G. Galli, N. Norais, D. Rosa, S. Campagnoli, V. Burgio, E. Di Stasio, B. Giardina, M. Houghton, S. Abrignani, and G. Grandi. 2000. Structure-function analysis of hepatitis C virus envelope-CD81 binding. *J. Virol.* 74:4824-4830.
- Pileri, P., Uematsu, Y., Campagnoli, S., Galli, G., Falugi, F., Petracca, R., Weiner, A. J., Houghton, M., Rosa, D., Grandi, G., and Abrignani, S. 1998. Binding of hepatitis C virus to CD81. *Science* 282[5390], 938-941.
- Prince, A. M., T. Huima-Byron, T. S. Parker, and D. M. Levine. 1996. Visualization of hepatitis C virions and putative defective interfering particles isolated from low-density lipoproteins. *J. Viral Hepat.* 3:11-17.

Ray SC, Wang YM, Laeyendecker O, Ticehurst JR, Villano SA, Thomas DL. 1999 Acute hepatitis C virus structural gene sequences as predictors of persistent viremia: hypervariable region 1 as a decoy. *J Virol.* Apr;73(4):2938-46

Ross RS, Viazov SO, Holtzer CD, Beyou A, Monnet A, Mazure C, Roggendorf M. Genotyping of hepatitis C virus isolates using CLIP sequencing. *J Clin Microbiol.* 2000 Oct;38(10):3581-4.

Saldanha J, Lelie N, Heath A. Establishment of the first international standard for nucleic acid amplification technology (NAT) assays for HCV RNA. WHO Collaborative Study Group. *Vox Sang.* 1999;76(3):149-58

Sasaki N, Matsui A, Momoi M, Tsuda F, Okamoto H. Loss of circulating hepatitis C virus in children who developed a persistent carrier state after mother-to-baby transmission. *Pediatr Res.* 1997 Sep;42(3):263-7.

Scarselli E, Ansuini H, Cerino R, Roccasecca RM, Acali S, Filocamo G, Traboni C, Nicosia A, Cortese R, Vitelli A. The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus *EMBO J.* 2002 Oct 1;21(19):5017-25.

Schmidt, W. N., Wu, P., Han, J. Q., Perino, M. J., Labrecque, D. R., and Stapleton, J. T. 1997. Distribution of hepatitis C virus (HCV) RNA in whole blood and blood cell fractions: Plasma HCV RNA analysis underestimates circulating virus load. *Journal of Infectious Diseases* 176[1], 20-26.

Scognamiglio P, Accapezzato D, Casciaro MA, Cacciani A, Artini M, Bruno G, Chircu ML, Sidney J, Southwood S, Abrignani S, Sette A, Barnaba V. 1999 Presence of effector CD8+ T cells in hepatitis C virus-exposed healthy seronegative donors. *J Immunol.* Jun 1;162(11):6681-9.

Shiffman, M. L., Hofmann, C. M., Contos, M. J., Luketic, V. A., Sanyal, A. J., Sterling, R. K., FerreiraGonzalez, A., Mills, A. S. , and Garret, C. 1999. A randomized, controlled trial of maintenance inteferon therapy for patients with chronic hepatitis C virus and persistent viremia. *Gastroenterology* 117[5], 1164-1172.

Shimizu, Y. K., Feinstone, S. M., Kohara, M., Purcell, R. H., and Yoshikura, H. 1996. Hepatitis C virus: Detection of intracellular virus particles by electron microscopy. *Hepatology* 23[2], 205-209.

Simmonds, P. 1993. Variability of hepatitis C virus genome. *In* H. W. Reesink (ed.), *Current studies in Haematology and blood transfusion; Hepatitis C.* Karger, Basel.

Simmonds, P., E. C. Holmes , T. A. Cha, S.-W. Chan, F. McOmish, B. Irvine, E. Beall, P. L. Yap, J. Kolberg, and M. S. Urdea. 1993. Classification of hepatitis C virus into six major genotypes and a series of subtypes by phylogenetic analysis of the NS-5 region. *J. Gen. Virol.* 74:2391-2399.

Simmonds, P., K. A. Rose, S. Graham, S. W. Chan, F. McOmish, B. C. Dow, E. A. C. Follett, P. L. Yap, and H. Marsden. 1993. Mapping of serotype-specific, immunodominant epitopes in the NS- 4 region of hepatitis C virus (HCV) - use of type-specific peptides to serologically differentiate infections with HCV type 1, type 2, and type 3. *J. Clin. Microbiol.* 31:1493-1503.

Simmonds, P., D. B. Smith, F. McOmish, P. L. Yap, J. Kolberg, M. S. Urdea, and E. C. Holmes. 1994. Identification of genotypes of hepatitis C virus by sequence comparisons in the core, E1 and NS-5 regions. *J. Gen. Virol.* 75:1053-1061.

Simons, J. N., T. P. Leary, G. J. Dawson, T. J. Pilot-Matias, A. S. Muerhoff, G. G. Schlauder, S. M. Desai, and I. K. Mushahwar. 1995. Isolation of novel virus-like sequences associated with human hepatitis. *Nature Med.* 1:564-569.

Smith, D. B., J. Mellor, L. M. Jarvis, F. Davidson, J. Kolberg, M. Urdea, P. L. Yap, P. Simmonds, J. D. Conradie, A. G. S. Neill, G. M. Dusheiko, M. C. Kew, R. Crookes, A. Koshy, C. K. Lin, C. Lai, I. M. Murraylyon, A. Elguneid, A. A. Gunaid, T. Yemen, S. Yemen, D. Mutimer, M. Ahmed, C. Nuchprayoon, S. Tanei R, Watanabe K, Nishiyama S 1995 Clinical and histopathologic analysis of the relationship between lichen planus and chronic hepatitis C. *J Dermatol.* 1995 May;22(5):316-23.

Tanprasert, F. E. Preston, M. Makris, A. Chuansumrit, C. Mahasandana, D. Pritchard, E. Riley, B. M. Greenwood, A. A. Saeed, A. M. Alrasheed, M. G. Saleh, I. Mcfarlane, C. Tibbs, R. Williams, J. Power, E. Lawlor, and H. Kiyokawa. 1995. Variation of the hepatitis C virus 5' non-coding region: implications for secondary structure, virus detection and typing. *J. Gen. Virol.* 76:1749-1761.

Tsai SL, Liaw YF, Chen MH, Huang CY, Kuo GC. 1997 Detection of type 2-like T-helper cells in hepatitis C virus infection: implications for hepatitis C virus chronicity. *Hepatology.* Feb;25(2):449-58.

Sartori M, La Terra G, Aglietta M, Manzin A, Navino C, Verzetti G. Transmission of hepatitis C via blood splash into conjunctiva *Scand J Infect Dis.* 1993;25(2):270-1

Shiffman ML, Hofmann CM, Thompson EB, Ferreira-Gonzalez A, Contos MJ, Koshy A, Luketic VA, Sanyal AJ, Mills AS, Garrett CT. Relationship between biochemical, virological, and histological response during interferon treatment of chronic hepatitis C. *Hepatology.* 1997 Sep;26(3):780-5.

Stuyver, L., R. Rossau, A. Wyseur, M. Duhamel, B. Vanderborght, H. Van Heuverswyn, and G. Maertens. 1993. Typing of hepatitis C virus isolates and characterisation of new subtypes using a line probe assay. *J. Gen. Virol.* 74:1093-1102.

Sugitani, M. and Shikata, T. 1998. Comparison of amino acid sequences in hypervariable region-1 of hepatitis C virus clones between human inocula and the infected chimpanzee sera. *Virus Res.* 56[2], 177-182.

Takahashi, K., S. Kishimoto, H. Yoshizawa, H. Okamoto, A. Yoshikawa, and S. Mishiro. 1992. p26-protein and 33-nm particle associated with nucleocapsid of hepatitis-c virus recovered from the circulation of infected hosts. *Virology* 191:431-434.

Takaki A, Wiese M, Maertens G, Depla E, Seifert U, Liebetrau A, Miller JL, Manns MP, Rehermann B. 2000 Cellular immune responses persist and humoral responses decrease two decades after recovery from a single-source outbreak of hepatitis C. *Nat Med.* 2000 May;6(5):578-82.

Tanaka, T., Kato, N., Cho, M. J., Sugiyama, K., and Shimotohno, K. 1996. Structure of the 3' terminus of the hepatitis C virus genome. *Journal of Virology* 70[5], 3307-3312.

Tanaka, T., K. Tsukiyamakohara, K. Yamaguchi, S. Yagi, S. Tanaka, A. Hasegawa, Y. Ohta, N. Hattori, and M. Kohara. 1994. Significance of specific antibody assay for genotyping of hepatitis C virus. *Hepatology* 19:1347-1353.

Thimme R, Oldach D, Chang KM, Steiger C, Ray SC, Chisari FV. 2001 Determinants of viral clearance and persistence during acute hepatitis C virus infection. *J Exp Med.* 2001 Nov 19;194(10):1395-406.

Thio CL, Nolt KR, Astemborski J, Vlahov D, Nelson KE, Thomas DL. Screening for hepatitis C virus in human immunodeficiency virus-infected individuals. *J Clin Microbiol.* 2000 Feb;38(2):575-7.

Thomssen, R., S. Bonk, C. Propfe, K. H. Heermann, H. G. Kochel, and A. Uy. 1992. Association of hepatitis c virus in human sera with beta- lipoprotein. *Med. Microbiol. Immunol. (Berl)* 181:293-300.

Thomssen, R., S. Bonk, and A. Thiele. 1993. Density heterogeneities of hepatitis C virus in human sera due to the binding of beta-lipoproteins and immunoglobulins. *Med. Microbiol. Immunol. (Berl)* 182:329-334.

Toyoda, H., Kumada, T., Nakano, S., Takeda, I., Sugiyama, K., Osada, T., Kiriya, S., Sone, Y., Kinoshita, M., and Hadama, T. 1997. Quasispecies nature of hepatitis C virus and response to alpha interferon: Significance as a predictor of direct response to interferon. *Journal of Hepatology* 26[1], 6-13.

Tsukiyama Kohara, K., N. Iizuka, M. Kohara, and A. Nomoto. 1992. Internal ribosome entry site within hepatitis C virus RNA. *J. Virol.* 66:1476-1483.

Tsukiyama Kohara, K., K. Yamaguchi, N. Maki, Y. Ohta, K. Miki, M. Mizokami, K. Ohba, S. Tanaka, N. Hattori, A. Nomoto, and M. Kohara. 1993. Antigenicities of group I and group II hepatitis C virus polypeptides - molecular basis of diagnosis. *Virology* 192:430-437.

Van Doorn, L. J. 1994. Detection and characterisation of the hepatitis C virus. Erasmus University, Rotterdam.

Vandoorn, L. J., B. Kleter, L. Stuyver, G. Maertens, H. Brouwer, S. Schalm, R. Heijntink, and W. Quint. 1994. Analysis of hepatitis C virus genotypes by a line probe assay and correlation with antibody profiles. *J. Hepatol.* 21:122-129.

Vrieling, H., C. L. Vanderpoel, H. W. Reesink, H. L. Zaaijer, E. Scholten, L. C. M. Kremer, H. T. M. Cuypers, P. N. Lelie, and M. H. J. Vanoers. 1995. Look-back study of infectivity of anti-HCV ELISA-positive blood components. *Lancet* 345:95-96.

Wang, J. T., J. C. Sheu, J. T. Lin, T. H. Wang, and D. S. Chen. 1992. Detection of replicative form of hepatitis-c virus RNA in peripheral blood mononuclear cells. *J. Infect. Dis.* 166:1167-1169.

Wasley A, Alter MJ. Epidemiology of hepatitis C: geographic differences and temporal trends *Semin Liver Dis.* 2000;20(1):1-16.

Weiner, A. J., H. M. Geysen, C. Christopherson, J. E. Hall, T. J. Mason, G. Saracco, F. Bonino, K. Crawford, C. D. Marion, K. A. Crawford, and et al. 1992. Evidence for immune selection of hepatitis C virus (HCV) putative envelope glycoprotein variants: potential role in chronic HCV infections. *Proc. Natl. Acad. Sci. USA* 89:3468-3472.

Wunschmann, S., J. D. Medh , D. Klinzmann, W. N. Schmidt, and J. T. Stapleton. 2000. Characterization of hepatitis C virus (HCV) and HCV E2 interactions with CD81 and the low-density lipoprotein receptor. *J. Virol.* 74:10055-10062.

Xu, L. Z., M. Martinotpeignoux, P. Marcellin, J. P. Benhamou, and D. Larzul. 1994. Comparison of the sensitivity of nested PCR in the 5' non- coding and the NS5 regions of the HCV genome. *J. Hepatol.* 20:598-602.

Yasui, K., Wakita, T., Tsukiyamakohara, K., Funahashi, S., Ichikawa, M., Kajita, T., Moradpour, D., Wands, J. R., and Kohara, M. The native form and maturation process of hepatitis C virus core protein. *Journal of Virology* 72[7], 6048-6055. 1998.

Yuan H, Shahidi A, Park S, Guilfoyle D and Hirshfield I. 2003. Detection of Extrahepatic Hepatitis C Virus Replication by a Novel, Highly Sensitive, Single-Tube Nested Polymerase Chain Reaction *Am J Clin Pathol* 119(1):95-100

Yuki, N., Hayashi, N., Moribe, T., Matsushita, Y., Tabata, T., Inoue, T., Kanazawa, Y., Ohkawa, K., Kasahara, A., Fusamoto, H., and Kamada, T. 1997. Relation of disease activity during chronic hepatitis C infection to complexity of hypervariable region 1 quasispecies. *Hepatology* 25[2], 439-444.

Yun, A. B., Lara, C., Johansson, B., deRivera, I. L., and Sonnerborg, A. 1996. Discrepancy of hepatitis C virus genotypes as determined by phylogenetic analysis of partial NS5 and core sequences. *J. Med Virol.* 49[3], 155-160.

Yun, Z. B., Barkholt, L., and Sonnerborg, A. 1997. Dynamic analysis of hepatitis C virus polymorphism in patients with orthotopic liver transplantation. *Transplantation* 64[1], 170-172.

Zein, N. N., J. Rakela, and D. H. Persing. 1995. Genotype-dependent serologic reactivities in patients infected with hepatitis C virus in the united states. *Mayo Clin. Proc.* 70:449-452.

Zhang, Z. X., Z. B. Yun, M. Chen, A. Sonnerborg, and M. Sallberg. 1995. Evaluation of a multiple peptide assay for typing of antibodies to the hepatitis C virus: relation to genomic typing by the polymerase chain reaction. *J. Med. Virol.* 45:50-55.

Zignego, A. L., D. Macchia, M. Monti, V. Thiers, M. Mazzetti, M. Foschi, S. Romagnani, P. Gentilini, and C. Brechot. 1992. Infection of peripheral mononuclear cells by hepatitis C virus. *J. Hepatol.* 15:382-386.

Appendix 1

Serum Extraction Mix Final Concentration

0.11M Sodium Chloride BDH Lutterworth UK

55mM Tris(hydroxymethyl)methylamine hydrochloride (Tris-HCl) BDH

1.1mM EDTA Kodak

0.5 % Sodium Dodecylsulphate Sigma Poole, Dorset

2mg/ ml Poly A Amersham Little Chalfont, UK

10mg/ ml Proteinase K Roche Lewes, UK

10 X TBE Solution

108g Tris Base

55g Boric acid

9.3 g Na₂ EDTA.2H₂O

Tris EDTA (TE) Solution pH8

10mM Tris -HCl

1mM EDTA

SOC Medium 100 ml pH 7

2g Bacto-Tryptone

0.5g Bacto-Yeast extract

1 ml 1M NaCl

0.25 ml 2M Mg²⁺ stock (1M NaCl₂.6H₂O)

Add Bacto-Tryptone, Bacto-yeast extract, NaCl and KCl to 97 ml distilled water. Stir to dissolve. Autoclave and cool to room temperature. Add 2M Mg²⁺ stock and 2M glucose, each to a final concentration of 20mM. Bring to 100 ml with sterile distilled water. Filter the complete medium through a 0.2µm filter unit.

Luria -Bertani Broth

10g/l Tryptone

5g/l yeast extract

5g/l NaCl

Adjust to pH 7 with NaOH

LB Agar

Add 15g agar to 1l of LB Broth

Glucose Tris Lysozyme GTE Solution

0.9g glucose

2 ml 0.5M EDTA

2.5 ml Tris-HCl

NaOH SDS Solution 10 ml

400 μ l 5M NaOH

1 ml 10%SDS

8.6 ml distilled water

KOH/ Glacial Acetic Acid Solution (Solution III)

29.5% Glacial Acetic acid

KOH pellets to pH 4.8

Appendix 2

Nucleotide sequences of the expressed clones from Core, NS3 and NS5 regions.
Numbering according to Choo et al., 1991.

Core genotype 1b

DX501 nucleotides 0-420

ATG AGC ACG AAT CCT AAA CCT CAA AGA AAA ACC AAA CGT AAC ACC AAC
CGC CGC CCA CAG GAC GTC AAG TTC CCG GGC GGT GGT CAG ATC GTT GGT
GGA GTT TAC TTG TTG CCG CGC AGG GGC CCC AGG TTG GGT GTG CGC GCG
ACT AGG AAG ACT TCC GAG CGG TCG CAA CCT CGT GGA AGG CGA CAA CCT
ATC CCC AAG GCT CGC CGG CCC GAG GGC AGG GCC TGG GCC CAG CCC GGG
TAT CCT TGG CCC CTC TAT GGC AAT GAG GGC ATG GGG TGG GCA GGA TGG
CTC CTG TCA CCC CGT GGC TCT CGG CCT AGT TGG GGC CCC ACG GAC CCC
CGG CGT AGG TCG CGT AAT CTG GGT AAG GTC ATC GAT ACC CTC ACA TGC
GGC TTC GGC GAC CTC ATG GGG TAC ATT CCG CTC GTC

Core genotype 3a

DX760 nucleotides 0-420

ATG AGC ACG AAT CCT AAA CCT CAA AGA AAA ACC AAA AGA AAC ACC ATC
CGT CGC CCA CAG GAC GTT AAG TTC CCG GGT GGC GGA CAG ATC GTT GGT
GGA GTA TAC GTG TTG CCG CGC AGG GGC CCA CGA TTG GGT GTG CGC ACG
ACG CGT AAA ACT TCT GAA CGG TCA CAG CCT CGC GAA CGG CGA CAG CCT
ATC CCC AAG GCG CGT CGG AGC GAA GGC CGG TCC TGG GCT CAG CCC GGG
TAC CCT TGG CCC CTC TAC GGT AAC GAG GGC TGC GGG TGG GCA GGG TGG
CTC CTG TCC CCG CGC GGC TCC CGT CCA TCT TGG GGC CAA AAT GAC CCC
CGG CGA AGG TCC CGC AAC TTG GGT AAA GTC ATC GAT ACC CTC ACG TGC
GGA TTC GCC GAC CTC GTG GGG TAC ATT CCG CTC GTA

Core genotype 4a

DX387 nucleotides 0-420

ATG AGC ACG AAT CCT AAA CCC CAA AGA AAA ACC AAA CGT AAC ACC AAC
CGC CGC CCA ATG GAC GTT AAG TTC CCG GGT GGT GGT CAG ATC GTT GGC
GGA GTT TAC TTG TTG CCG CGC AGG GGC CCC CGG TTG GGT GTG CGC GCG
ACT CGG AAA ACT TCG GAG CGG TCG CAA CCT CGT GGA AGA CGC CAG CCT
ATC CCC AAG GCG CGT CGA TCC GAG GGA AGG TCC TGG GCA CAA CCA GGA
TTT GCA TGG CCC CTT TAC GGT AAT GAG GGT TGC GGG TGG GCA GGA TGG
CTC TTG TCC CCC CGT GGT TCT CGA CCG TCT TGG GGC CCA AAT GAT CCC
CGG CGG AGG TCC CGC AAT TTG GGT AAA GTC ATC GAT ACT CTA ACC TGC
GGC CTC GCC GAC CTC ATG GGG TAC ATT CCG CTC GCC

NS3 genotype 1b

DX458 nucleotides 4079-4361

CCC AAC AUC GAG GAG GUG GCU CUG UCC AAC ACU GGA GAG AUC CCC UUC
UAU GGC AAA GCC AUC CCU AUU GAG ACC AUC AAG GGG GGG AGG CAC CUC
AUU UUC UGC CAC UCC AAG AAG AAG UGU GAC GAA CUC GCU GCA AAA CUG
GUG GGC CUC GGU AUC AAU GCU GUA GCG UAU UAC CGG GGC CUU GAU GUG
UCC GUC AUA CCG GCC AGC GGA GAC GUC GUU GUU GUA GCA ACA GAC GCU
CUA AUG ACG GGC UUU ACC GGC GAC UUU GAC UCA GUG AUC GAC UGU

NS3 genotype 3a

DX506 nucleotides 4079-4361

CCU AAC AUU GAG GAG GUG GCC CUG GGU UCU GAA GGU GAG AUC CCU UUC
UAC GGC AAG GCU AUA CCA AUA GCC CUG CUC AAG GGG GGG AGG CAC CUU
AUC UUU UGC CAU UCC AAG AAA AAG UGC GAU GAG AUA GCA UCC AAA CUC
AGA GGC AUG GGG CUC AAC GCU GUA GCA UAC UAU AGA GGU CUC GAU GUG
UCC GUC AUA CCA ACA ACA GGG GAU GUC GUA GUU UGC GCU ACU GAC GCC
CUC AUG ACU GGA UUC ACU GGG GAC UUC GAC UCC CUG AUC GAC UGU

NS3 genotype 4a

DX499 nucleotides 4079-4361

CCU AAC AUU GAG GAG GUC GCC CUG CCA ACA ACG GGG GAA AUA CCC UUU
UAC GGC AAG GCG AUC CCU CUG GAG CUG AUU AAG GGG GGC AGA CAU CUC
AUC UUC UGC CAC UCA AAG AAA AAG UGU GAU GAA CUG GCC AGA CAA CUG
ACA UCU CUU GGU CUG AAU GCC GUA GCC UAC UAC AGA GGC UUA GAC GUU
UCG GUG AUU CCC ACG UCU GGG GAC GUC GUG GUA UGC GCC ACG GAC GCC
CUC AUG ACG GGU UUU ACC GGC GAC UAU GAC UCC GUC AUC GAC UAC

NS5 genotype 3a

DX507 nucleotides 6712-6961

GGA TCC AAT ATT ACT CGC GTG GAG TCT GAA ACA AAG GTT GTG ATT CTT
GAT TCG TTC GAA CCC CTG AGA GCC GAA ACT GAC GAC GCC GAG CTC TCA
GTG GCT GCG GAG TGT TTG AAG AAA CCT CCC AAG TAC CCT CCA GCT CTT
CCT ATT TGG GCT AGG CCA GAT TAC AAT CCT CCA CTA TTG GAC CGC TGG
AAA GCA CCG GAT TAT GTA CCA CCA ACT GTC CAT GGG TGT GCC TTG CCA
CCA CAA GCT

Appendix 3

Derivation of the formula to calculate the percentage of type-specific and type-common antibody response.

Assume:

1. Reactivity of a type A serum for a type A antigen is 1 unit.

$$E_{AA}=1$$

2. The same is true for a type B serum with type B antigen.

$$E_{BB}=1$$

3. The reactivity equals the type-common and type-specific components

$$T_C + T_S = 1$$

4. The reactivity of a type B serum for a type A antigen is a product of the overall "antigenicity" times the type-common component.

$$E_{BA} = A_{AB} \times T_C$$

5. The antigenicity A_{AB} is the antigenicity of a genotype A antigen relative to the antigenicity of a genotype B antigen.

6. Reactivity of a sample of type B for type B antigen compared to reactivity of a sample of a type B for type A antigen

$$\frac{E_{BB}}{E_{BA}} = \frac{1}{A_{AB} \times T_C}$$

7. Reactivity of a sample of type A for antigen of type B compared to reactivity of a sample of type A for type A antigen

$$\frac{E_{BA}}{E_{AA}} = \frac{T_C}{A_{AB}}$$

Therefore:

$$\frac{E_{BB}/E_{BA}}{E_{AB}/E_{AA}} = \frac{1}{A_{AB} \times T_C} \times \frac{A_{AB}}{T_C}$$

And:

$$\frac{E_{BB}/E_{BA}}{E_{AB}/E_{AA}} = \frac{1}{(T_C)^2}$$

From this the proportion of type-common reactivity can be derived as follows:

$$T_C = \sqrt{\frac{E_{AB}/E_{AA}}{E_{BB}/E_{BA}}}$$

Antigenic Variation of Core, NS3, and NS5 Proteins among Genotypes of Hepatitis C Virus

J. A. NEVILLE,¹ L. E. PRESCOTT,¹ V. BHATTACHERJEE,¹ N. ADAMS,¹ I. PIKE,² B. RODGERS,²
A. EL-ZAYADI,³ S. HAMID,⁴ G. M. DUSHEIKO,⁵ A. A. SAEED,⁶
G. H. HAYDON,⁷ AND P. SIMMONDS^{1*}

Department of Medical Microbiology, University of Edinburgh,¹ and Department of Medicine, University of Edinburgh, Royal Infirmary of Edinburgh,⁷ Edinburgh, Murex Biotech Ltd., Dartford, Kent,² and Department of Medicine, Royal Free Hospital, London,⁵ United Kingdom; Cairo Liver Centre, Dokki, Giza, Egypt³; Department of Medicine, Aga Khan University, Karachi, Pakistan⁴; and Department of Pathology, Riyadh Armed Forces Hospital, Riyadh, Saudi Arabia⁶

Received 18 April 1997/Returned for modification 1 July 1997/Accepted 19 August 1997

Assays that detect antibody to hepatitis C virus (HCV) are used to screen blood donors and patients with hepatitis. Current enzyme-linked immunosorbent assay (ELISA)-based methods are invariably based upon antigens from expressed recombinant proteins or oligopeptides from HCV type 1. Some HCV antigens used in screening assays are coded by regions of the HCV genome that show extensive variability; therefore, HCV type 1-based assays may be less effective for the detection of antibody elicited by infection with other genotypes. In this study, we have measured antibody reactivity of sera from 110 hepatitis C patients infected with type 1b, 3a, or 4a to genotype-specific and cross-reactive epitopes present in recombinant proteins from HCV genotypes 1b (core, NS3, and NS5), 3a (NS3, NS5), and 4a (core, NS3), corresponding to those used in current third-generation screening ELISAs. By comparing the serological reactivities of sera to type-homologous and type-heterologous antigens, we detected a significant type-specific component to the reactivity to NS3 (61 to 77% of the total reactivity) and NS5 (60% of the total reactivity). Furthermore, despite the similarities in the amino acid sequences of the core antigens of type 1b and type 4a, we also found significantly greater reactivity to type-homologous antigens, with approximately 25% of reactivity being type specific. These findings are consistent with previous findings of fivefold weaker reactivity of sera from HCV type 2- and HCV type 3-infected blood donors in the currently used third-generation ELISAs and suggest that these assays are suboptimal for screening populations in which the predominant genotype is not type 1.

Detection of antibody to hepatitis C virus (HCV) has become the principal method for the diagnosis of HCV infection in individuals with chronic hepatitis and for the screening of blood donors. Although the original assay based upon the c100-3 recombinant proteins derived from NS4 showed non-specificity and insensitivity, the more recently developed assays that use recombinant proteins from the core and NS3 regions of the HCV genome (second-generation assays) and the NS5 region of the HCV genome (third-generation assays) have proved to be more effective for the screening of blood donors. Their use has led to a substantial reduction in the incidence of posttransfusion hepatitis. In prospective studies, the incidence of HCV transmission among recipients of blood screened by first-generation assays was 1.5% in Spain (15), 3.7% in Japan (27), and 11% in Taiwan (6). Screening by second-generation assays reduced or would have reduced the incidence to 0.9% in Japan (27), 1% in Spain (15), 2% in Greece (20), and 2.5% in Taiwan (6).

One reason for the failure to detect antibody to HCV in donated blood specimens that transmit HCV infection is that the blood was collected from an individual with acute infection before seroconversion for antibody. This so-called "window" period is long for HCV compared with other viruses for which blood from donors is screened, such as human immunodeficiency virus, with means of 88 to 66 days in second- and third-

generation assays, respectively (9). By measuring the incidence of HCV infection in blood donors, it has been estimated that the current residual risk of HCV transmission through collection of "window" samples is 1 per 100,000 donations in the United States (24). This frequency of infection is similar to the residual risk of infection from blood screened by third-generation assays from French blood donors (10).

In addition, samples false negative for antibody have been reported for a small proportion of immunocompetent individuals persistently infected with HCV (2, 17). Indeed, even in anti-HCV-positive individuals, there is considerable variability in the frequency of reactivity to the individual HCV proteins used in supplementary assays, such as the Ortho 3rd generation recombinant immunoblot assay (RIBA-3), and in the titer of antibody to HCV among persistently infected individuals. Among 90 anti-HCV-positive blood donors, antibody reactivity in the Ortho third-generation screening assay varied over a range of 5,000-fold, with some serum samples having antibody levels only just above the cutoff for the assay (12).

HCV can be classified into at least six major genotypes, whose nucleotide and inferred amino acid sequences over the whole genome differ by approximately 30%. This degree of amino acid sequence variability is similar to that observed between variants of other RNA viruses (e.g., dengue virus types 1 to 4), in which significant antigenic differences have been documented and which form the basis of their classification into serotypes. We have previously measured the serological reactivities of individuals infected with different HCV genotypes to antigens used in two third-generation anti-HCV assays (Ortho 3rd generation anti-HCV EIA; Murex VK48).

* Corresponding author. Mailing address: Department of Medical Microbiology, University of Edinburgh, Teriot Place, Edinburgh, United Kingdom. Phone: 44 131 650 3138. Fax: 44 131 650 6531. E-mail: Peter.Simmonds@ed.ac.uk.

Samples from HCV type 1-infected individuals showed an approximately five times greater reactivity than those infected with HCV type 2 or 3, and the reactivity was independent of other factors that may have influenced the antibody response, such as the degree of viremia, donor age, and severity of hepatitis as assessed by alanine aminotransferase measurements.

In the current study we have addressed two of the main shortcomings of the first study. First, we have measured serological reactivities to the individual component antigens used in a third-generation assay (core, NS3, and NS5). These measurements extend previous investigations of type-specific and cross-reactive serological reactivity to the NS4 antigen used in the first-generation screening assays and in second- and third-generation confirmatory assays (1, 5, 14, 21, 26, 28). Second, we have carried out titrations with antigens derived from HCV type 1 as well as corresponding proteins from other HCV genotypes (types 3a and 4a). This allows levels of antibodies to both type-homologous and type-heterologous antigens to be measured, allowing for a more rigorous assessment of the relative levels of type-specific and type-common reactivity to each.

MATERIALS AND METHODS

Samples. Samples LJ516, EG21, and ED43 were obtained from individuals infected with genotype 3a (LJ516) or 4a (EG21 and ED43 [3, 25]). Sequences amplified from the core, NS3, and NS5 regions of types 3a and 4a were used for the synthesis of antigens for antibody screening. Recombinant antigens from the core (amino acid positions 1 to 140), NS3 (amino acid positions 1360 to 1454), and NS5 (amino acid positions 2234 to 2318) (numbered according to Choo et al. [7]) regions of HCV-UK (type 1b) were derived from an existing, commercially available anti-HCV assay (VK48).

Serum samples were obtained from 110 anti-HCV-positive individuals with chronic hepatitis C attending hospital liver clinics in Edinburgh and London, United Kingdom; Karachi, Pakistan; and Cairo, Egypt. Among the samples, we selected 33 infected with genotype 1b, 34 infected with genotype 3a, and 43 infected with genotype 4a. Genotypes were identified by restriction fragment length polymorphism analysis of the 5' noncoding region as described previously (11).

Development of enzyme-linked immunosorbent assays (ELISAs) based upon type 3a and 4a antigens. RNAs from samples LJ516 (type 3a), EG21 (type 4a), and ED43 (type 4a) were extracted as described previously (16). Reverse transcription was carried out with avian myeloblastosis virus reverse transcriptase and RNasin (both from Promega, Southampton, United Kingdom), in each case by using the external antisense primer, and incubation at 42°C for 30 min as described previously (4). The sequences were amplified by using the following heat cycle: 94°C for 18 s, 45°C for 21 s, and 72°C for 90 s for 30 cycles, followed by 6 min at 72°C with the primers listed in Table 1. The core region of type 4a was amplified with primers 954 and 410, followed by amplification with primers D236 and 597. The NS3 region was amplified with primers 751 and 753, followed by amplification with primers 594 and 593 (type 3a) or 750 and 007 and then primers 594 and 593 (type 4a). For the type 4a NS3 region, a third PCR was carried out with the product of the second PCR by using the same inner primers. The NS5 region of genotype 3a was amplified by using primers 991 and 993, followed by amplification with primers 3155 and 3156. The amplified sequences were cloned and sequenced as described previously (23).

Expression of recombinant proteins. The sequences of the core, NS3, and NS5 regions from the different HCV genotypes indicated above were subcloned into *Escherichia coli* or baculovirus expression vectors, as appropriate, by standard molecular biology techniques. The NS3 and NS5 sequences of each genotype were cloned into the *E. coli* vector pTrcHis (Invitrogen BV, Leek, The Netherlands) and were expressed in TOP10 cells by standard isopropyl- β -D-thiogalactopyranoside induction. Core sequences were cloned into the baculovirus expression vector pBlueBacHis2 (Invitrogen), and recombinant virus was derived by cotransfection into insect cells along with linear *Antographa californica* nuclear polyhedrosis virus DNA. Recombinant antigens were produced in Sf9 insect cells in an LH Series 210 bioreactor by infection at multiplicity of infection of 2 for 48 h.

Both the *E. coli* and baculovirus vector expression vectors place a tract of six histidine residues at the amino terminus of each recombinant protein. Recombinant proteins were therefore subsequently prepared by metal chelate chromatography on Probond nickel affinity resin (Invitrogen) to approximately 90% purity.

ELISA for detection of antibody to core, NS3, and NS5 regions. To optimize plate coating, purified antigens were titrated to levels giving acceptable specificity and sensitivity and approximately equivalent amounts of protein per well (25 to

TABLE 1. Primers used for amplification of core, NS3, and NS5 regions

Primer no.	Sequence ^a
954.....	ACT GCC TGA TAG GGT GCT TGC GAG
410.....	GAG CGG AAT GTA CCC CAT GAG GTC GGC
D236.....	GGT AGA CCG GAT CCC ATG AGC ACG AAT CCT AAA
597.....	GAA AGA AGC TTA GAC GAG CGG AAT GTA CCC
751.....	TTY CGG GCN GCT GTG TGC ACC
753.....	GCG TCA TAG CAC TCA CAG AGG AC
594.....	ACC RCT GCA GAY CCY AAC ATN GAG GAR GT
593.....	CAC AAG CTT ARC AGT AGT CKA TSA CNG ART C
750.....	TGG TYA CNN GNN AYG CYG ATG TCA TYC C
007.....	ACC TCG AGT ATC CCA CTG ATG AAG TTC CAC AT
991.....	TGG CNA GCT CNT CNG CNA GCC A
993.....	GGC TCC CCC TCA AGG GRG GCA TRG A
3155.....	GGA TCC AAY ATN ACY CGY GTR GAG TCW SA
3156.....	AAG CTT GTG GTG GYA AGG CAC AYC C

^a International Union of Pure and Applied Chemistry ambiguity codes were used: Y, C/T; R, A/G; N, G/C/A/T; K, G/T; S, C/G; W, A/T. Cleavage sites for restriction enzymes are underlined: CTGCAG, *Pst*I; AAGCTT, *Hind*III; GGA TCC, *Bam*HI.

50 ng). Binding of antigen was assessed both by functional enzyme immunoassay (EIA) with positive and negative sera and by probing for the histidine tag present on each protein by using horseradish peroxidase chemically modified to bind nickel ions. All antigens were coated overnight in 50 mM Tris (pH 8.5) containing 0.02% sodium dodecyl sulfate. The plates were then blocked by the addition of 2% degraded gelatin and were finally dried to ensure stability.

For each antigen assay, each serum sample was diluted 1:10, 1:40, 1:160, and 1:640 in anti-HCV-negative sera. Aliquots of 20 μ l from each titration were added to 180 μ l of sample diluent, mixed, and then added to a well of the ELISA plate. A solution of 1:50 *E. coli* blocker was added to the diluent for all assays for NS3 genotype 3a antigen. The plates were then incubated at 37°C for 1 h, followed by five washes in glycerate-borate buffer. Bound antibody was detected by the addition of 100 μ l of horseradish peroxidase-labelled antibody to human immunoglobulin G conjugate and by incubation for 30 min at 37°C. After washing, 100 μ l of 3,3',5,5'-tetramethylbenzidine substrate was added, and the mixture was incubated for 30 min at 37°C. The color development reaction was stopped by the addition of 50 μ l of 2 M H₂SO₄, and optical densities (ODs) were measured at 470 nm. All assay plates included a dilution series of the reference genotype 1a sample (dilutions of 1:2 to 1:2,560) for calculation of antibody levels. The NS3 genotype 3a recombinant antigens were expressed in *E. coli* and required the addition of 1:50 *E. coli* blocking solution in the sample diluent for all assays.

Quantification of antibody levels. Antibody reactivity to each recombinant protein in each of the test serum samples was determined by titration and reference to a standard positive control as described previously (12). This method is based upon the observation of a linear relationship between OD (over the range of values of 0.02 to 1.0) and concentration of antibody in both ELISAs based upon single antigens, as in the current study and in the currently used third-generation screening assays from Ortho and Murex (12). Because of this relationship, the antibody reactivity of a test sample can be expressed relative to that of a reference control by the following formula: test antibody level = (test OD \times reference concentration)/(reference OD \times test concentration). Antibody levels were obtained for each sample in each antigen assay in this way.

Estimation of relative proportions of type-specific and cross-reactive antibodies. The ratio of antibody levels measured to type-homologous and type-heterologous antigens indicates the relative proportions of type-specific and cross-reactive reactivities. This calculation is complicated by the possible existence of differences in the antigenicity or presentation of proteins from different genotypes in the ELISA and differences in antibody levels elicited by infection with different genotypes.

The proportion of antibody reactivity that is cross-reactive between genotypes is measured by reciprocal assays of reactivity between sera and antigens from two genotypes, genotypes A and B. In the following relations, E_{BA} represents the antibody reactivity of a serum sample infected with genotype B with antigen of genotype A, A_{AB} is the overall antigenicity of the genotype A antigen relative to that of the genotype B antigen, and T_c and T_s are the proportions of the antibody response that are type common and type specific, respectively, such that $T_c + T_s = 1$.

$$\frac{E_{BB}}{E_{BA}} = \frac{E_{BB}}{E_{BB} \times A_{AB} \times T_c} = \frac{1}{A_{AB} \times T_c}$$

$$\frac{E_{AB}}{E_{AA}} = \frac{E_{AA} \times (1/A_{AB}) \times T_c}{E_{AA}} = \frac{T_c}{A_{AB}}$$

A	1	21	41	61	70		
1a HCV-PT	MSTNPKPQKK	NKRNTNRRPQ	DVKFPGGGQI	VGGVYLLPRR	GPRLGVRATR	KTSESRQPRG	RRQPIPKARR
1a HC-J1	...I...R.	T.....V..
1b HCV-JR.	T.....
1b BKR.	T.....P.
HCVUPCPR.	T.....Q
1c HC-J9R.	T.....V.....
2a HC-J6R.	T.....D..
DX503P2AR.	T.....T..Q.....D..
2b HC-J8R.	T.....D..
2c BEBE1R.	T.....A.....D..
3a NZL1	...L...R.	T...I....V....
3a HPCHK6	...L...R.	T...I....	YV...V....K.....
3b Tr	...L...KRQ	T...L...K	N...A....	...E...V....	...Q...EV..T...P
10a JK049	...L...RI	T...I....V....	...K...V....S	...R...
4a ED43R.	T.....M
DX387C4AR.	T.....M
11a JK046RQ	T.....
DX461C5PR.	T.....D.Q
	71	91	111	131	140		
1a HCV-PT	PEGRTWAQPG	YPWPLYGNEG	CGWAGWLLSP	RGSRPSWGPT	DPRRRSRNLG	KVIDTLTCGF	ADLMGYIPLV
1a HC-J1
1b HCV-J	M.....
1b BK	L.....
DXUPCP	...A.....	M.....
1c HC-J9	...S.....S
2a HC-J6	ST.KS.GK..	L.....N	...H...V.V.
DX503	STDKS.GK..	L.....H...V.V.
2b HC-J8	ST.KS.GK..T...	...H...R...	...I....V.
2c BEBE1	ST.KS.GR..	...R...	L.....S	...HK....V.
3a NZL1	S...S.....N
3a HPCHK6	S...S.....N.A.N
3b Tr	R...S.....P.	...QNI
10a JK049	T...S.....N
4a ED43	...S.....N	...G....
DX387	S...S.....	FA.....	...NL
11a JK046	QT...A.G...	...A.....	...T...NLVI
DX461	...T...S.G...	...A...L	...N...N	...K....

FIG. 1. Comparison of the inferred amino acid sequences of antigens expressed from recombinant clones of (A) core (HCVUPCP, type 1b; DX387, type 4a), (B) NS3 (HCVUPCP, type 1b; DX506, type 3a; DX499, type 4a), and (C) NS5 regions (HCVUPCP, type 1b; DX507, type 3a) with representatives of each of the major HCV genotypes and subtypes. Periods indicate identity with the HCV prototype sequence, HCV-PT (7). Numbers refer to amino acid positions on HCV-PT.

Therefore,

$$\frac{E_{BB}/E_{BA}}{E_{AB}/E_{AA}} = \frac{A_{AB}}{A_{AB} \times (T_c)^2} = \frac{1}{(T_c)^2}$$

From this the proportion of type-common reactivity can be derived as follows:

$$T_c = \sqrt{\frac{E_{AB}/E_{AA}}{E_{BB}/E_{BA}}}$$

and

$$A_{AB} = \sqrt{\frac{1}{(E_{AA}/E_{AB}) \times (E_{BA}/E_{BB})}}$$

These relations make the assumption that the relative proportions of type-specific to type-common reactivity in a type A antiserum are the same as those found in a type B antiserum.

Nucleotide sequence accession numbers. The sequences obtained in the course of this project have been submitted to GenBank and bear the following

accession numbers: DX387, AF029298; DX506, AF029297; DX499, AF029296; DX507, AF029299.

RESULTS

Variability of core, NS3, and NS5 region sequences. The amino acid sequences of the recombinant proteins used in the ELISA were deduced from the nucleotide sequences of the corresponding clones and were compared with other published sequences of these regions of HCV (Fig. 1). In the core protein only 9 amino acids differed between genotypes 1b and 4a over the 140-amino-acid length (6% divergence). Substitutions were generally conservative, where only one resulted in a change in the ionic charge. The NS3 sequences exhibited more variability, with the sequence of genotype 1b differing from that of genotype 3a by 15 of 95 amino acids (16% divergence) and from that of genotype 4a by 13 amino acids, while genotypes 3a

B		1360	1370	1380	1390	1400	1410	1420	1430	1440	1450
1a HCV-PT	PNIEEVALST	TGEIPFYGKA	IPLEVIKGR	HLIFCHSKKK	CDELAALVA	LGINAVAYR	GLDVSVIPTS	GDVVVVATDA	LMTGYTGDFD	SVIDC	
1a HC-J1	A.....A.....V.....	
1b HCV-JN.....I.A.....TG.....L.....F.....	
1b BKN.....I.A.R.....SG.....I.....	
HCVUPCPN.....I.T.....G.....A.....F.....	
1c HC-J9	S.....	E.....NY.....G.....V.....F.....T.....	
2a HC-J6GQ.....	E.....R.....SY.....A.RG.....	M.L.....Q.....F.....	
2b HC-J8	S.....GH.....	E.....AF.....A.RG.....	M.V.....Q.....	
2c BEBE1GH.....	E.....SA.....VA.RG.....	M.L.....I.....Q.....	
3a NZL1	S.....GS.....	E.....IALL.....I.S.....RG.....	M.L.....T.....C.....F.....	
3a HPCHK6	S.....GS.....	E.....IAC.....KM.S.....RG.....	M.L.....T.....C.....F.....	
DX506GS.....	E.....IALL.....I.S.....RG.....	M.L.....T.....C.....F.....	Y
3b TrG.TS.....	D.....L.AM.....V.....E.....S.....RG.....	M.V.....F.....V.....C.....T.....	
10a JK049	S.....TG.....	E.....R.....G.....KQ.TS.....V.....F.....Q.....C.....I.....	
4a ED43	S.....P.....L.....RQ.TS.....L.....C.....F.....	
DX499P.....L.....RQ.TS.....L.....C.....F.....Y.....	Y
11a JK046	...Q.T...PL...	...V.....	...Y.....Q.RT...	...L...F...	...V.....	...C.....	

C		2234	2244	2254	2264	2274	2284	2294	2304	2314
1a HCV-PT	GGNITRVESE	NKVVILDSFD	PLVAEEDE-R	EISVPAEILR	KSR-RFAQAL	PVWARPDYNP	PLVETWKKPD	YEPPVVHGCP	LPPPK	
1a HC-J1T.....I.....I.....N.....Q.....	
1b HCV-JIR.V.....P.K.PP.....I.....L.S.D.....V.....ST.....	
1b BKR.....V.....K.K.PA.M.....I.....L.S.D.....V.....I.....	
HCVUPCPV.....R.....V.....K.K.PA.M.....I.....L.S.D.....V.....I.....	
1c HC-J9I.....D.....L.....K.K.PP.M.....I.....P.R.....L.....	
2a HC-J6DV.....I.....	S.....V.....L.....	M.E.RSD.L	P.I.S.YML	PKK.....PP.....	A.....S.R.....	Q.AT.A.A	
2b HC-J8DV.....I.....D.....	S.....IV.....L.....	SMTEV.D.D.	P.I.S.Y.I	R.K.PP.....	P.....	V.I.....R.G.....T.L.A.....	TP	
2c BEBE1M.....I.....	S.....LMV.....	V.DK.....	P.I.S.Y.L	PKS.....PP.....	P.....L.....R.....	Q.....A.A.....	G	
3a NZL1S.....	T.....V.....E.....	R.R.T.D.V	P.A.A.CFK	PP.KYPP.....	I.....LDR.A.A.....	V.T.A.....	RG	
3a HPCHK6S.....	T.....	E.....R.R.T.D.A	L.AA.CFK	PP.KYPP.....	I.....LDR.S.....	V.T.A.....	KG	
DX507S.....	T.....	E.....R.R.T.D.A	L.A.A.C.K	PP.KYPP.....	I.....LDR.A.A.....	V.T.A.....	QA	
3b TrS.....	T.....	E.....R.R.D.T	L.I.CFK	PP.KYPP.....	I.....LPS.D.T.....	A.A.....	TR	
10a JK049S.....	S.....	E.....R.CD.D	L.A.A.CFK	PP.KYPP.....	I.....P.D.....	V.T.A.....	Q	
4a ED43STA.....TD.....	E.....I.....E.....	SC.QNDD	V.A.....	PTK.K.PP.....	I.....T.....QQ.....	QA.T.A.....	A	
11a JK046I.....	E.....K.....F.D.....A.....CH.....	PP.FKYPP.....L.....A.....	D.....S.A.....	QG	

FIG. 1—Continued.

and 4a differed from each other by 14 amino acids. In the NS5 sequence there were 23 differences between genotypes 1b and 3a over a length of 85 amino acids (27% divergence). Six of these substitutions affected the charge of the protein: four substitutions of nonpolar amino acids for basic groups and two substitutions of acidic groups for nonpolar groups.

Quantitation of antibody reactivity to core, NS3, and NS5 antigens. The serological reactivities of sera from 33 individuals with genotype 1b, 34 individuals with genotype 3a, and 43 individuals with genotype 4a HCV infections to each of the recombinant antigens from genotypes 1b, 3a, and 4a were measured. To investigate the reproducibility of the method used to quantify antibody levels, each of the serum specimens was assayed in replicate against the type 1b and 4a core proteins (Fig. 2). A close correlation was observed between the two measured antibody levels, with nonparametric correlation coefficients being 0.877, 0.889, and 0.862 for type 1b, 3a, and 4a antisera, respectively. For the type 4a core antigens, the cor-

responding correlation coefficients were 0.784, 0.721, and 0.802.

There was also a close correlation between reactivity to the core protein of type 1b and that to type 4a (Table 2), with correlation coefficients of 0.834, 0.765, and 0.767 for type 1b, 3a, and 4a antisera, respectively. Significant correlations were also consistently observed between the reactivities of antisera to NS3 proteins of types 1b, 3a, and 4a, as well as between the reactivities of antisera to NS5 proteins of type 1b and 3a. In contrast, there was little if any correlation between antibody reactivity to different regions of the genome. For example, the reactivity of sera from type 1b-infected individuals to the type 1b core protein showed no correlation with reactivity to type 1b NS3 or NS5 proteins (correlation coefficients, -0.118 and -0.181, respectively; Table 2). The only exceptions were weak correlations (0.443 and 0.471) between the reactivities to 3a NS3 and NS5 (but only for type 1b sera) and between NS5 of type 1b with NS3 of 4a (restricted to type 3a sera).

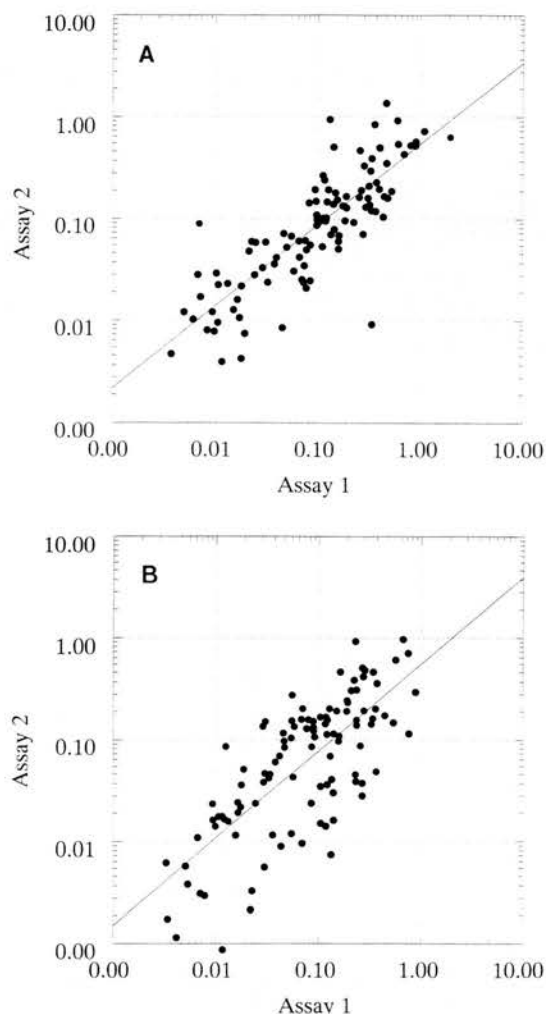


FIG. 2. Measurement of the reproducibility of measurement of levels of antibody to type 1b (A) and type 4a (B) core protein by sera from study subjects by repeat testing. The correlation is indicated by a regression line.

Genotype dependence of serological reactivity. Sera collected from individuals infected with different genotypes varied in their frequency of reactivity to NS3 and NS5 antigens of different genotypes (Table 3). For NS3, frequencies of reactivity for type 1b, 3a, and 4a antisera to type-homologous NS3 proteins were 85, 100, and 76%, respectively, compared with a range of 44 to 76% for type-heterologous combinations. Similarly, the frequency of reactivity of type 1b sera with type 1b NS5 antigen (65%) was higher than that of heterologous sera (47 and 51%), as was reactivity to type 3a NS5 (58% type-homologous reactivity, compared with 24 and 45% for type-heterologous combinations). In the core region, high frequencies of reactivity were observed for both type-homologous (95 to 97%) and type-heterologous (86 to 97%) combinations, indicating the greater antigenicity of this region of the genome and/or a greater proportion of shared epitopes between genotypes.

To compare the strength of reactivity with type-homologous and type-heterologous antigens, antibody levels relative to those for the positive control calculated from OD readings at dilutions ranging from 1:10 to 1:640 were used to calculate antibody levels relative to those for the positive control (Fig. 3). There were significant differences in antibody reactivity to

the type 1b core protein between samples from individuals infected with genotype 1b and those from individuals infected with genotype 4a ($P = 0.033$) but not those with genotype 3a ($P = 0.811$). The distribution of antibody levels directed to the genotype 4a core antigen showed no significant difference between genotypes.

Antibody reactivity against NS3 was frequently undetectable among sera from individuals infected with HCV with type-heterologous antigen (Table 3; Fig. 3C). The distribution of levels of antibody to type-homologous antigens was consistently greater than those to type-heterologous antigens. For example, the median level of antibody to type 1b NS3 in type 1b sera was 0.225, which was substantially greater than the median reactivities of type 3a and 4a sera to this protein (0.003 [$P = 0.004$] and 0.02 [$P = 0.005$], respectively). Similar, predominant type-specific reactivities against type 3a and 4a antigens were observed (Fig. 3D and E).

Only 54% of the samples reacted with the NS5 genotype 1b antigen and 43% reacted with the genotype 3a antigen. The median antibody level of genotype 1b sera (0.031) was eightfold higher than that for type 3a samples (0.004) and threefold higher than that for type 4a (0.012). The distribution of antibody levels among genotype 1b samples was significantly higher than that among genotype 4a samples ($P = 0.029$) but not genotype 3a samples. The reactivity of type 3a sera against

TABLE 2. Correlation between reactivity to different antigens

Antigen and genotype	HCV genotype in serum	Correlation for the following antigens and genotypes ^a :					
		Core		NS3			NS5
		1b	4a	1b	3a	4a	1b 3a
Core 1b	1b	(1)					
	3a	(1)					
	4a	(1)					
4a	1b	<u>0.834</u>	(1)				
	3a	<u>0.765</u>	(1)				
	4a	<u>0.767</u>	(1)				
NS3 1b	1b	-0.118	0.023	(1)			
	3a	0.133	0.279	(1)			
	4a	0.100	-0.035	(1)			
3a	1b	-0.270	-0.066	<u>0.456</u>	(1)		
	3a	0.153	0.274	<u>0.363</u>	(1)		
	4a	0.159	0.030	<u>0.627</u>	(1)		
4a	1b	0.048	0.224	<u>0.710</u>	<u>0.518</u>	(1)	
	3a	0.215	0.130	<u>0.570</u>	<u>0.393</u>	(1)	
	4a	-0.058	-0.087	<u>0.497</u>	0.272	(1)	
NS5 1b	1b	-0.181	-0.220	0.150	0.275	0.157	(1)
	3a	0.066	0.000	0.218	0.265	<u>0.471</u>	(1)
	4a	0.058	-0.192	0.069	0.168	0.055	(1)
3a	1b	-0.141	-0.43	0.247	<u>0.443</u>	0.348	<u>0.630</u> (1)
	3a	0.051	0.172	0.216	0.266	0.202	<u>0.649</u> (1)
	4a	-0.076	-0.108	0.186	0.179	0.041	0.303 (1)

^a Correlation is indicated by Spearman's rank correlation coefficient. Separate values are provided for antisera of each genotype. Significant values ($P < 0.05$) are underlined.

TABLE 3. Frequency of reactivity to core, NS3, and NS5 proteins

Antiserum	No. of specimens reactive to the following antigen/total no. of specimens (%):						
	Core		NS3			NS5	
	1b	4a	1b	3a	4a	1b	3a
Type 1b	32/33 (97)	32/33 (97)	28/33 (85)	25/33 (76)	24/32 (76)	21/33 (64)	8/33 (24)
Type 3a	33/34 (97)	30/34 (88)	15/34 (44)	33/33 (100)	9/33 (27)	16/34 (47)	18/31 (58)
Type 4a	37/43 (86)	41/43 (95)	31/43 (72)	27/43 (63)	31/41 (76)	22/43 (51)	19/42 (45)

NS3 of genotype 3a was significantly greater than that of the type 1b or 4a sera ($P = 0.002$ and 0.01 , respectively).

Ratio of type-specific and cross-reactive reactivities. The ability to measure the reactivity of the same antiserum to

antigens of different genotypes allowed for an estimation of the relative levels of genotype-specific and cross-reactive serological reactivities. For the core proteins, the levels of antibody to the genotype 1b antigen in each of the type 1b and 4a sera were

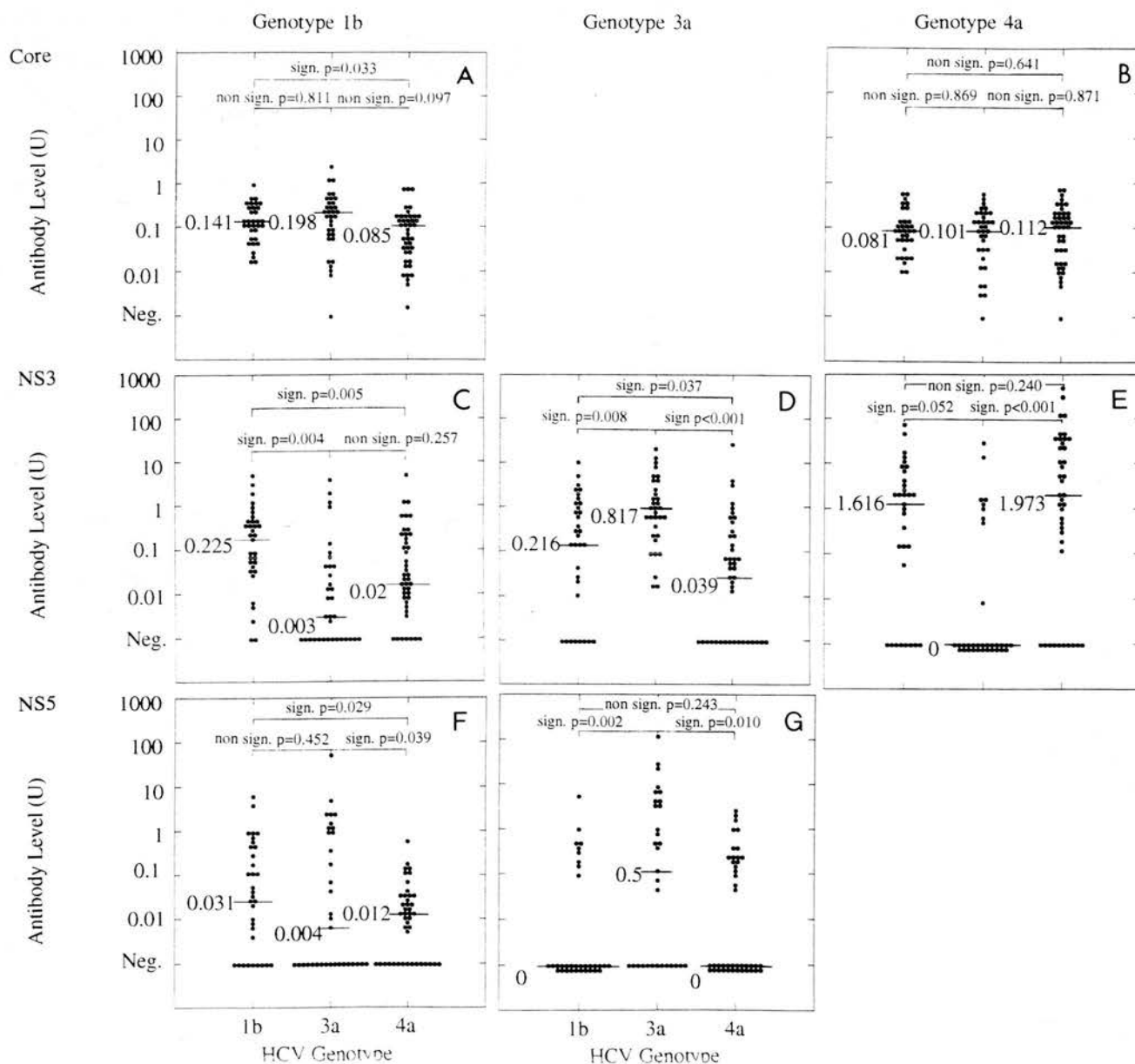


FIG. 3. Distribution of antibody reactivity of sera from individuals infected with type 1b, 3a, and 4a to core proteins of genotypes 1b and 4a (A and B, respectively); NS3 of genotypes 1b, 3a, and 4a (C, D, and E respectively); and NS5 of types 1b and 3a (F and G, respectively). Median values are indicated by short horizontal bars. Pairwise comparison of the distributions of values was carried out by the nonparametric Kruskal-Wallis test.

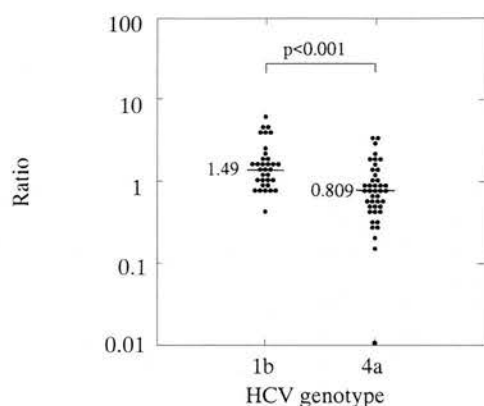


FIG. 4. Ratios of reactivity of type 1b and 4a sera to type 1b/type 4a core antigens. Median ratios are indicated by short horizontal bars. Pairwise comparison of the distributions of values was carried out by the Kruskal-Wallis test. Ratios of reactivities to other antigens are presented in Table 4.

divided by their levels to the genotype 4a antigen (Fig. 4; Table 4). The median ratio of reactivity to type 1b/reactivity to type 4a antigens for type 1b antisera was 1.49, compared with a median ratio of 0.809 for the type 4a antisera. This indicates that type 1b sera react more strongly against the type 1b (homologous) core protein, while the type 4a sera react slightly more strongly against the type 4a antigen. If it were shown that both core proteins were coated equivalently on the solid phase and showed equal antigenicity, then the type-specific reactivity to these proteins could be calculated from the difference from the ratio of 1 that would be expected from exclusively type-common reactivity. However, it is possible that some antigens are present at higher available concentrations than others through differences in binding to the solid phase or solubility. These differences were taken into account by using the derivation described in Materials and Methods. In this instance, the term E_{AB}/E_{AA} represents the median of the ratio of reactivity between type 4a antisera with type 1b and type 4a antigens (0.809). The term E_{BB}/E_{BA} is similarly represented by the median ratio of the type 1b antisera (1.49). Therefore, the proportion of type-common reactivity can be calculated as 0.74, with type-specific reactivity forming the remainder of the reactivity (0.26).

The median ratio of reactivity of the type 3a sera to type 3a and 1b NS5 antigens (0.654) was substantially lower than that of type 1b sera (median, 4; Table 4). Therefore, the proportions of type-common and type-specific reactivity were 40 and 60%, respectively; i.e., the majority of the serological reactivity to this antigen was type specific. Finally, three sets of pairwise comparisons can be made for the NS3 region, in which antigens for all three genotypes were available. Sixty-two percent of the serological reactivity between type 1b and 3a proteins was type specific, similar to the proportions of 61% between type 1b and 4a proteins and 77% between type 3a and 4a proteins (Table 4).

DISCUSSION

Antigenic variability of HCV. The aim of this study was to investigate the degree of type-specific serological reactivity to antigens used in current, third-generation screening assays. The use of recombinant antigens expressed from different genotypes in the enzyme immunoassay allowed reciprocal measurements of type-homologous and type-heterologous reactivity to be made, and these provided a more rigorous assessment

of the type-specific components of reactivity to the core, NS3, and NS5 regions. This addresses a potential criticism of previous investigations that showed weaker reactivity of sera from individuals infected with non-type 1 genotypes in either of the screening ELISAs or to individual antigens in the confirmatory recombinant immunoblot assay (5, 8, 12, 14, 21, 28), in that there is a possibility that these observations resulted from a generally weaker serological response to infection than that elicited by type 1. In the current study we were able to consistently show stronger reactivity of sera to antigens of a homologous type than to antigens of heterologous types.

From pairwise reciprocal measurements of antibody reactivity (such as type 1b and 4a antisera against type 1b and 4a antigen), it was possible to quantify the relative contributions of type-specific and cross-reactive antibody reactivities by using the relation derived in Materials and Methods (Table 4). These calculations were independent of possible differences in the strength of the serological response elicited by infection with different genotypes and were also independent of differences in antigen concentration or overall antigenicity between proteins of different genotypes (expressed as the ratio A_{AB}). This removes the potential criticism that the coating efficiency of antigens from different genotypes onto the solid phase was not compared prior to measurement of antibody levels. From this analysis we found a relationship between the degree of amino acid sequence divergence between recombinant proteins and their degree of cross-reactivity. The sequence of the core protein was the most conserved and the core protein showed approximately 25% type-specific reactivity, while the more divergent sequences, those of the NS3 and NS5 regions, showed substantially greater proportions of type-specific reactivity. These results are consistent with previous comparisons of the type-specific component of reactivity to peptides corresponding to linear epitopes in NS4. By absorption in solution with peptides of heterologous genotypes, it was shown that reactivity to type-homologous peptides was reduced but was rarely eliminated (1, 26), allowing development of a sensitive and specific serological typing assay. The type-specific component of serological reactivity to NS4 and, in some studies, to the core protein has been also used in typing assays without cross-absorption, because it has consistently been observed that reactivity to type-homologous antigens in these (13, 19, 22) and other regions (29) is stronger.

Implications for screening assays. The effect of the demonstrated antigenic variabilities of the components of third-gen-

TABLE 4. Type-specific and type-common serological reactivity to HCV core, NS3, and NS5 antigens

Region	Anti-serum		Median ratio ^a		P value ^b	Percent		
	A	B	A	B		T_c	T_s	Amino acid divergence ^c
Core	4a	1b	0.809	1.49	<0.001	74	26	6.4
NS5	3a	1b	0.654	4.0	0.014	40	60	27
NS3	3a	1b	0.053	0.373	0.001	38	62	16
	4a	1b	0.016	0.107	<0.001	39	61	14
	4a	3a	0.049	0.939	<0.001	23	77	15

^a Ratio of reactivity to antigens of genotypes A and B.

^b Comparison of the distribution of ratios of reactivity of individual antisera to antigens of genotypes A and B by the Kruskal-Wallis nonparametric test.

^c Degree of amino acid sequence divergence between antigens of genotypes A and B.

eration screening assays on their overall sensitivity for screening is difficult to estimate with precision for two reasons. Individuals vary in their serological responses to different antigens, and therefore in the extent to which these responses may result in cross-reactivity with antigens of heterologous genotypes. For example, if reactivity were directed solely to the core protein, this protein would cross-react with antigens of heterologous genotypes, whereas sera monoreactive with NS3, such as is found upon seroconversion, would be expected to be predominantly type specific.

Furthermore, each of the three antigens investigated is likely to contain a range of linear and conformational epitopes, and these will vary in their degree of cross-reactivity. Recognition of different epitopes in the antigens may be one explanation for the wide range of ratios of reactivity to type-homologous and type-heterologous antigens observed between sera of the same genotype (Fig. 4). In the extreme case, it is possible that reactivity confined to epitopes in the core protein that are type specific would lead to poor or absent reactivity to the core protein of other genotypes. Conversely, the high degree of cross-reactivity observed between certain sera with NS3 or NS5 proteins of heterologous genotypes may have resulted from their recognition of shared epitopes.

The observed differences in reactivity of sera to homologous and heterologous proteins would only lead to false-negative results on serological screening if antibody levels in samples to be tested were close to the cutoff sensitivity of the assay. In a previous study, we measured antibody reactivity in the Ortho third-generation assay and found a wide range of antibody levels (approximately 5,000-fold) among samples from blood donors infected with genotypes 1, 2, and 3, with the lowest level found to be 0.0007, which is just above the cutoff value of the test (12). Low antibody levels and reactivity to a restricted range of epitopes, such as those in NS3, are found in acutely infected individuals (18), and it is likely that earlier detection of seroconversions, and therefore a reduction in the window period associated with non-type 1 infection, may be achieved by assays containing NS3 and other antigens from a wider range of genotypes.

The finding of significant antigenic variability of antigens used for serological screening will form the basis for a number of future investigations. Now that antigens from other genotypes have been produced, it will be possible to carry out large-scale screening of populations infected with non-type 1 genotypes (for example, with the type 4a antigens for testing individuals in the Middle East). This may reveal the frequency with which anti-HCV samples are being missed by conventional assays and may ultimately help reduce the frequency of posttransfusion hepatitis further, particularly when populations with a high frequency of acute infection are screened. Similarly, the incorporation of antigens from other genotypes (particularly NS3) in confirmatory assays may resolve the results for a number of the indeterminate samples identified in blood donor screening and for which interpretation of results is currently problematic.

ACKNOWLEDGMENTS

We acknowledge the staff of the Hepatitis Reference Laboratory, Department of Medical Microbiology, University of Edinburgh, for assistance in providing facilities for serological testing and sample storage and provision. D. B. Smith provided the intellectual stimulus for the data analysis.

REFERENCES

- Bhattacharjee, V., L. E. Prescott, I. Pike, B. Rodgers, H. Bell, A. R. Elzayadi, M. C. Kew, J. Conradie, C. K. Lin, H. Marsden, A. A. Saeed, D. Parker, P. L. Yap, and P. Simmonds. 1995. Use of NS-4 peptides to identify type-specific antibody to hepatitis C virus genotypes 1, 2, 3, 4, 5 and 6. *J. Gen. Virol.* 76:1737-1748.
- Cerino, A., M. Bissolati, A. Cividini, A. Nicosia, M. Esumi, N. Hayashi, K. Mizuno, R. Slobbe, P. Oudshoorn, E. Silini, M. Asti, and M. U. Mondelli. 1997. Antibody responses to the hepatitis C virus E2 protein: relationship to viraemia and prevalence in anti-HCV seronegative subjects. *J. Med. Virol.* 51:1-5.
- Chamberlain, R., N. Adams, E. A. B. McCruden, P. Simmonds, and R. M. Elliott. 1997. The complete genome sequence of hepatitis C virus type 4a. *J. Gen. Virol.* 78:1342-1347.
- Chan, S.-W., F. McOmish, E. C. Holmes, B. Dow, J. F. Peutherer, E. Follett, P. L. Yap, and P. Simmonds. 1992. Analysis of a new hepatitis C virus type and its phylogenetic relationship to existing variants. *J. Gen. Virol.* 73:1131-1141.
- Chan, S. W., P. Simmonds, F. McOmish, P. L. Yap, R. Mitchell, B. Dow, and E. Follett. 1991. Serological responses to infection with three different types of hepatitis C virus. *Lancet* 338:1391.
- Chang, T. T., K. C. Young, Y. J. Yang, K. A. Lai, H. L. Wu, M. H. Wu, M. Y. Chen, X. Z. Lin, C. Y. Lin, and J. S. Shin. 1996. Incidence of post-transfusion hepatitis in Taiwan before and after introduction of anti-HCV testing. *Liver* 16:201-206.
- Choo, Q. L., K. H. Richman, J. H. Han, K. Berger, C. Lee, C. Dong, C. Gallegos, D. Coit, R. Medina Selby, P. J. Barr, A. J. Weiner, D. W. Bradley, G. Kuo, and M. Houghton. 1991. Genetic organization and diversity of the hepatitis C virus. *Proc. Natl. Acad. Sci. USA* 88:2451-2455.
- Claeys, H., A. Volckaerts, W. Mertens, Z. Liang, P. Fiten, and G. Opdenaker. 1995. Localization and reactivity of an immunodominant domain in the NS3 region of hepatitis C virus. *J. Med. Virol.* 45:273-281.
- Courouce, A. M., N. Lemarrec, A. Girault, S. Ducamp, and N. Simon. 1994. Anti-hepatitis C virus (anti-HCV) seroconversion in patients undergoing hemodialysis: comparison of second- and third-generation anti-HCV assays. *Transfusion* 34:790-795.
- Courouce, A. M., and J. Pillonel. 1996. Transfusion-transmitted viral infections. *N. Engl. J. Med.* 335:1609-1610.
- Davidson, F., P. Simmonds, J. C. Ferguson, L. M. Jarvis, B. C. Dow, E. A. C. Follett, C. R. G. Seed, T. Krusius, C. Lin, G. A. Medgyesi, H. Kiyokawa, G. Olim, G. Duraisamy, T. Cuypers, A. A. Saeed, D. Teo, J. Conradie, M. C. Kew, M. Lin, C. Nuchaprayoon, O. K. Ndimbie, and P. L. Yap. 1995. Survey of major genotypes and subtypes of hepatitis C virus using RFLP of sequences amplified from the 5' non-coding region. *J. Gen. Virol.* 76:1197-1204.
- Dhaliwal, S. K., L. E. Prescott, B. C. Dow, F. Davidson, H. Brown, P. L. Yap, E. A. C. Follett, and P. Simmonds. 1996. Influence of viraemia and genotype upon serological reactivity in screening assays for antibody to hepatitis C virus. *J. Med. Virol.* 48:184-190.
- Dixit, V., S. Quan, P. Martin, D. Larson, M. Brezina, R. Dinello, K. Sra, J. Y. N. Lau, D. Chien, J. Kolberg, A. Tagger, G. Davis, A. Polito, and G. Gitnick. 1995. Evaluation of a novel serotyping system for hepatitis C virus: strong correlation with standard genotyping methodologies. *J. Clin. Microbiol.* 33:2978-2983.
- Dow, B. C., H. Munro, I. Buchanan, E. A. C. Follett, F. Davidson, P. L. Yap, and P. Simmonds. 1996. Third-generation recombinant immunoblot assay: comparison of reactivities according to hepatitis C virus genotype. *Transfusion* 36:547-551.
- Gonzalez, A., J. I. Esteban, P. Madoz, L. Viladomiu, J. Genesca, E. Muniz, J. Enriquez, X. Torres, J. M. Hernandez, J. Quer, X. Vidal, H. J. Alter, J. W. Shih, R. Esteban, and J. Guardia. 1995. Efficacy of screening donors for antibodies to the hepatitis C virus to prevent transfusion-associated hepatitis: final report of a prospective trial. *Hepatology* 22:439-445.
- Jarvis, L. M., H. G. Watson, F. McOmish, J. F. Peutherer, C. A. Ludlam, and P. Simmonds. 1994. Frequent reinfection and reactivation of hepatitis C virus genotypes in multitransfused hemophiliacs. *J. Infect. Dis.* 170:1018-1022.
- Kao, J. H., M. Y. Lai, Y. T. Hwang, P. M. Yang, P. J. Chen, J. C. Sheu, T. H. Wang, H. C. Hsu, and D. S. Chen. 1996. Chronic hepatitis C without anti-hepatitis C antibodies by second-generation assay: a clinicopathologic study and demonstration of the usefulness of a third-generation assay. *Dig. Dis. Sci.* 41:161-165.
- Lelie, P. N., H. T. M. Cuypers, H. W. Reesink, C. L. van der Poel, I. Winkel, E. Bakker, P. J. Vanexelohlers, D. Vallari, J. P. Allain, and L. Mimms. 1992. Patterns of serological markers in transfusion-transmitted hepatitis-C virus infection using 2nd-generation HCV assays. *J. Med. Virol.* 37:203-209.
- Machida, A., H. Ohnuma, F. Tsuda, E. Muneoka, T. Tanaka, Y. Akahane, H. Okamoto, and S. Mishiro. 1992. Two distinct subtypes of hepatitis C virus defined by antibodies directed to the putative core protein. *Hepatology* 16:886-891.
- Makris, K., V. Kouvelis, I. Drakopoulos, E. Oikonomou, and A. Maniatis. 1995. Frequency and characteristics of post-transfusion hepatitis in Greece with emphasis on hepatitis C: comparing second- and third-generation assays. *Transfus. Med.* 5:213-224.
- McOmish, F., S.-W. Chan, B. C. Dow, J. Gillon, W. D. Frame, R. J. Craw-

- ford, P. L. Yap, E. A. C. Follett, and P. Simmonds. 1993. Detection of three types of a C virus in blood donors: investigation of type-specific differences in serological reactivity and rate of alanine aminotransferase abnormalities. *Transfusion* **33**:7-13.
22. Mondelli, M. U., A. Cerino, F. Bono, A. Cividini, A. Maccabruni, M. Aricò, A. Malfitano, G. Barbarini, V. Piazza, L. Minoli, and E. Silini. 1995. Hepatitis C virus (HCV) core serotypes in chronic HCV infection. *J. Clin. Microbiol.* **32**:2523-2527.
23. Prescott, L. E., A. Berger, J. M. Pawlotsky, P. Conjeevaram, I. Pike, and P. Simmonds. Sequence analysis of hepatitis C virus variants producing discrepant results with two different genotyping assays. *J. Med. Virol.*, in press.
24. Schreiber, G. B., M. P. Busch, S. H. Kleinman, and J. J. Korelitz. 1996. The risk of transfusion-transmitted viral infections. *N. Engl. J. Med.* **334**:1685-1690.
25. Simmons, P., F. McOmish, P. L. Yap, S. W. Chan, C. K. Lin, G. Dusheiko, A. A. Saeed, and E. C. Holmes. 1993. Sequence variability in the 5' non coding region of hepatitis C virus: identification of a new virus type and restrictions on sequence diversity. *J. Gen. Virol.* **74**:661-668.
26. Simmonds, P., K. A. Rose, S. Graham, S. W. Han, F. McOmish, B. C. Dow, E. A. C. Follett, P. L. Yap, and H. Marsden. 1993. Mapping of serotype-specific, immunodominant epitopes in the NS-4 region of hepatitis C virus (HCV): use of type-specific peptides to serologically differentiate infections with HCV type 1, type 2, and type 3. *J. Clin. Microbiol.* **31**:1493-1503.
27. Takano, S., K. Nakamura, S. Kawai, O. Yokosuka, Y. Satomura, and M. Omata. 1996. Prospective assessment of donor blood screening for antibody to hepatitis C virus by first- and second-generation assays as a means of preventing posttransfusion hepatitis. *Hepatology* **23**:708-712.
28. Zein, N. N., J. Rakela, and D. H. Persing. 1995. Genotype-dependent serologic reactivities in patients infected with hepatitis C virus in the United States. *Mayo Clin. Proc.* **70**:449-452.
29. Zhang, Z. X., Z. B. Yun, M. Chen, A. Sonnerborg, and M. Sallberg. 1995. Evaluation of a multiple peptide assay for typing of antibodies to the hepatitis C virus: relation to genomic typing by the polymerase chain reaction. *J. Med. Virol.* **45**:50-55.